ACUTE NECROTIZING ULCERATIVE GINGIVITIS: MICROBIAL AND IMMUNOLOGIC STUDIES

ANNUAL REPORT

William A. Falkler, Jr., Ph.D.

October 1983

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This is an annual report on an ongoing research project aimed at obtaining new information as to the microbial etiology and immunopathology of acute necrotizing ulcerative gingivitis (ANUG). Thirty one patients have been studied as of the date of this report. Completed patient history forms have showed individuals with the disease display a typical and similar life style. They do not have sound employment, they are not financially stable, they smoke and do not display normal living patterns. Subgingival plaque samples taken from the patients revealed the presence of large numbers of spirochetes and

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Gram - rods and cultural studies have demonstrated the presence of 8-12 different microorganisms in the lesion with members of the genera Bacteroides and Fusobacterium in the highest numbers. Tests were performed which displayed the characteristic hemagglutination activity of the F. nucleatum and B. gingivalis isolates. Both type I and II colonial variants of F. nucleatum were isolated from the ANUG lesion and serological studies with the F. nucleatum isolates from patients with ANUG, chronic periodontitis, juvenile periodontitis, and adults and children with healthy gingiva suggest that irregardless of which disease they were isolated from the microorganisms share antigenic determinants when reacted with human serum and rabbit antiserum. The reaction of sera from ANUG patients and age and sex matched healthy individuals with microbial isolates from the ANUG patients revealed no differences in the levels of IgG, IgA and IgM or the IgG antibody activity. The results also suggest that antigenic determinants are shared by the Fusobacterium nucleatum isolates and the Bacteroides gingivalis isolates. Electron microscopic observation of the types of spirochetes present in the ANUG plaque samples with regards to the axial filament arrangement suggested the predominant type displayed the "2-4-2" axial filament arrangement however "6-12-6", "8-16-8" and "12-24-12" arrangements were observed. Scanning electron microscopy of an extracted tooth from an ANUG lesion showed the predominant flora to be of spirochetes. Ten strains of spirochetes have been isolated representing "2-4-2" axial arrangement. Rabbit antisera are being prepared to these isolates for serologic characterization. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the ANUG isolates as compared to known spirochetal strains suggested the ANUG isolates displayed distinct different gel patterns from other T. denticola strains. Studies were undertaken to determine if microorganisms isolated from ANUG lesions have specific binding receptors for corticosteroids. The results suggested that B. gingivalis has specific receptors for dexamethasone and cortisol whereas F. nucleatum and B. intermedius do not. Biopsies of the diseased tissues from two of the patients were obtained and the histopathologic studies suggest that a PMN infiltration is seen early after onset whereas the gingival tissue is infiltrated with lymphocytes as time after onset of the lesion increases.

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ACUTE NECROTIZING ULCERATIVE GINGIVITIS

A Brief Literature Review

In 1896 Vincent described an ulcerative infection of the gingival tissue which he believed to be caused by fusiform bacilli and spirochetal organisms (1). Orban (2) utilized the term acute necrotizing ulcerative gingivitis (ANUG) to describe this infection and his description follows. The patient usually presents with painful hemorrhagic gingivae, inability to partake of food or to brush his teeth, and often with general malaise. He may or may not be aware of increased salivation, a noisome odor and a metallic taste. There is ulceration and necrosis of the interdental papillae with possible extension to the marginal gingivae. Craters may be present with the loss of interdental papillae. The crateriform lesions are commonly covered with pseudomembranes and surrounded by erythematous borders. Fever and lymphadenopathy are common.

The etiology of the disease has not followed Koch's postulates in that exudate from an infected lesion has not produced ANUG lesions in humans and animals. Although fusospirochetal abscesses have been produced in experimental animals, the lesions are not similar to those of ANUG (2-5). Clinical lesions have been produced in animals only after first traumatizing the involved tissues (6-8). Other investigators have mentioned the importance of local factors such as erupting teeth, poor oral hygiene, poor margins on restorations, calculus build-up, poor contacts, occlusal factors and systemic factors such as physical exhaustion, emotional tension, nutritional deficiencies and metabolic disturbances (2,3,5,9-13).

Of these, emotional factors often appear before the onset of the disease regardless of the other factors (11,12,14,15). Although there have been epidemics of ANUG, the disease has been proven to be noncommunicable. The epidemics were due to the fact that the individuals affected were under the same adverse conditions, such as mental stress or altered living patterns (2,4,5,16-19). This disease has been well documented in military personnel (14,20,21).

Treatments of the disease have utilized chromic acid, nitrates TCA, H_2O_2, sodium permanganate, mercurials, arsenicals and triamicinolene acetonide in an adhesive vehicle (4,9,18,22,23). Antibiotics have been and are still used as adjuncts to complete subgingival curettage, debridement and local corrective procedures (19-33). Also ultrasonic instruments have been used in routine periodontal procedures (34-37).

Early studies on the histopathology of ANUG lesions reported that the pathological process appears to begin on the surface of the epithelium. First, the keratin is destroyed, then the degeneration progressed through the various layers of the epithelium (including the basal layer and continues on into the immediate fibrous tissues). The adjacent tissues become edematous and infiltrated with inflammatory cells. On the surface of the lesion were found spirochetes, fusiform bacilli, cocci, and filamenting types (38-43). Spirochetes of large and intermediate size were shown capable of invading non-necrotic tissue of the ANUG lesion and the majority of these spirochetes appeared different from pure strains of cultivated Borrelia vincentii and Treponema microdentium (42). Electron microscopic observation of ANUG lesions allowed the observation of a bacterial zone containing numerous microorganisms, including various morphological types of spirochetes, a zone rich in neutrophiles, a zone of necrosis, a zone where larger spirochetes were observed within the tissues of the host in large numbers and to the exclusion of other organisms (42).

The participation of spirochetes in the etiology of fusospirochetal diseases still remains undetermined. Rosebury et al. (44-46) in a series of experiments using mixtures of 29 bacterial cultures and five spirochetal cultures were unable to elicit infections in guinea pigs different than that observed when using fusospirochetal exudate (46). Sixteen bacterial strains recombined with Treponema microdentium did not produce fusospirochetal abscesses in guinea pigs (47). Typical "fusospirochetal" abscesses have been observed in guinea pigs with a mixture of two strains of Bacteroides, a motile gram-negative rod and diphtheroid (48). In this system neither spirochetes nor fusiform bacteria were essential. Borrelia vincenti, Borrelia buccale and small oral treponemes

produced localized abscesses in rabbits (49). Spirochetal abscesses were also observed in the hamster cheek pouch after injection of Borrelia buccalis and small oral treponemes (50). Intracutaneous lesions which resulted in abscess formation were routinely produced in rabbits with both Fusobacterium nucleatum and Fusobacterium polymorphum alone or in combination with oral spirochetes. The synergistic combinations of fusobacteria and spirochetes in intracutaneous lesions in animals showed definite evidence of invasion of both types of organisms into the surrounding tissues (51). Sections of interdental papilla of Vincent's infection stained by the Warthin-Faulkner method revealed spirochetes and fusiform bacilli penetrating the tissue (52).

Cell-mediated immunity and humoral antibody studies were investigated in patients with acute ulcerative gingivitis using antigens from Actinomyces viscosus, Fusobacterium fusiform, Veillonella alcalescens and Bacteriodes melaninogenicus. No difference in serum antibody levels reactive to the antigens were observed between patients and controls. The significantly greater cell-mediated immunity to F. fusiforme in ANUG suggested this organism might be involved in the change from the chronic to the acute form of the disease (53).

Immunologic studies of ANUG were undertaken to detect humoral antibody reactive with oral spirochetes and to ascertain if spirochetes in vivo were coated with IgG, IgA, IgM and C3. Sera taken at the acute stage of ANUG revealed low antibody titers to oral spirochetes and ranged from 0 to 80. Also in ANUG, spirochetes in smears from the lesions were coated in vivo with IgG, IgA, IgM and C3. The authors suggested these findings are indicative of a localized antibody production to oral spirochetes. It was suggested that the interaction of these antibodies with spirochetes and subsequent complement activation may contribute to the pathogenesis of ANUG (54).

Jacob et al. (55) isolated anaerobic spirochetes from patients and prepared rabbit antisera. The antisera detected. with the use of an indirect fluorescent antibody technique, oral spirochetes in dental plaque from 10 patients suggesting that a common antigenic determinant was shared by the spirochetes detected. These investigators have

also isolated from oral spirochetes a sodium deoxycholate-ethanol extractable antigen which appears to be shared by several oral isolates and to which human antibody is reactive (56).

Loesche et al. (57) anaerobically cultured plaque samples from 22 ulcerated sites in eight patients with ANUC. They observed a constant flora comprised of a limited number of bacterial types and a variable flora composed of a heterogeneous mixture of bacterial types. The constant flora included Treponema and Selenomonas sp., B. intermedius and Fusobacterium sp. Treatment with metronidazole resulted in a prompt resolution of clinical symptoms with a significant reduction in the numbers of Treponema sp., B. intermedius and Fusobacterium sp. for several months following treatment.

SUMMARY

This is an annual report on an ongoing research project aimed at obtaining new information as to the microbial etiology and immunopathology of acute necrotizing ulcerative gingivitis (ANUG). Thirty one patients have been studied as of the date of this report. Completed patient history forms have showed individuals with the disease display a typical and similar life style. They do not have sound employment, they are not financially stable, they smoke and do not display normal living patterns. Subgingival plaque samples taken from the patients revealed the presence of large numbers of spirochetes and Gram - rods and cultural studies have demonstrated the presence of 8-12 different microorganisms in the lesion with members of the genera Bacteroides and Fusobacterium in the highest numbers. Tests were performed which displayed the characteristic hemagglutination activity of the F. nucleatum and B. gingivalis isolates. Both type I and II colonial variants of F. nucleatum were isolated from the ANUG lesion and serological studies with the F. nucleatum isolates from patients with ANUG, chronic periodontitis, juvenile periodontitis, and adults and children with healthy gingiva suggest that irregardless of which disease they were isolated from the microorganisms share antigenic determinants when reacted with human serum and rabbit antiserum. The reaction of sera from ANUG patients and age and sex matched healthy individuals with microbial isolates from the ANUG patients revealed no differences in the levels of IgG, IgA and IgM or the IgG antibody activity. The results also suggest that antigenic determinants are shared by the Fusobacterium nucleatum isolates and the Bacteroides gingivalis isolates. Electron microscopic observation of the types of spirochetes present in the ANUG plaque samples with regards to the axial filament arrangement suggested the predominant type displayed the "2-4-2" axial filament arrangement however "6-12-6", "8-16-8" and "12-24-12" arrangmenets were observed. Scanning electron microscopy of an extracted tooth from an ANUG lesion showed the predominant flora to be of spirochetes. Ten strains of spirochetes have been isolated representing the "2-4-2" axial

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ANUG PATIENTS
Clinical Evaluation and History

As of this time 31 patients with ANUG have been studied. The clinical evaluation of the patients is shown in Table 1.

The patients were asked to fill out an "ANUG History" questionaire. The answers were as follows:

1. What is your present job:

   Thirty two percent of the patients were unemployed at the time of coming to the clinic. The following jobs were listed:

   a. food service
   b. hair stylist
   c. barmaid
   d. electronics
   e. operations manager
   f. students
   g. file clerk
   h. service station manager
   i. bakery worker
   j. clerk
   k. dancer
   l. dental hygienist
   m. insurance broker
   n. family counselor

2. Are you satisfied with your present job?

   Only 35% were satisfied with their present job.

3. Living conditions:

   58% lived at home with their family
   16% lived alone
   25% lived in an apartment with a roommate

4. Fifty one percent of the patients were male; forty nine per cent were female

5. The mean age of the patients was 23 with a range of 14 to 50.

6. The average length of time that they lived at their current address was 54 months with a range of 1 week to 27 years.

7. The patients classified their own health status as follows:

   22% Excellent
   56% Good
   22% Fair

8. They came to the dental clinic because:

   62.5% Painful, bleeding gums
   8.3% Painful gums
   4.1% Painful tongue
   8.3% Other

9. Seventy five percent of the patients had sore gums when they entered the dental clinic.

Table 1
ANUG Patients

Patient #	Gingival Bleeding S	Gingival Bleeding P	Blunting of Papilla	Loss of Papilla 1/3, 1/3-2/3, 2/3	Pain	Fetid Odor	Cratering of Papilla	Pseudo-membrane	Lymph-adenopathy	Onset	Temp.	Area Affected
1	+		+	+	+	+	+	+	+	2 weeks	99.2	Mand. Anterior
2		+	+	+	+	-[a]	+	+	-[a]	1 month	--[c]	Mand. Ant. Max. Post
3	+		+	+	+	+	+	+	+	--	--	Generalized
4		+	--[a]	+	+	+	+	±[b]	+	5 months	--	Max. Ant. Mand. Ant.
6	+		+		+	+	+	+	--	--	--	Mand. Ant.
7		+	--	+	+	+	±	±	--	3 days	--	Mand. Ant.
8	+		+	+	+	+	+	+	--	3 days	--	Mand. Ant.
9	+		--		+	+	+	+	--	1 week	--	Max. Ant. Manx. Post.
10		+	--		+	+	+	+	+	1 week	99.6	Generalized
11	+		+	+	+	+	+	+	--	5 days	--	Generalized
12		+	±[b]	+	+	+	+	±	--	1 week	--	Generalized
13	+		--	+	+	+	+	+	+	1 week	--	Mand. Ant. Max. Ant.
14		+	--	+	+	+	±	±	--	4 days	--	Mand. Ant. Mand. Post.
15	+		+	+	+	+	+	+	--	4 days	--	Mand. Ant.
16	+		--	+	+	+	+	+	+	3 days	--	Mand. Ant.

17	+		+		+		+	+	+	+	--	2 weeks	--	Mand. Ant.
18		+ +	--	+			+	+	+	+	--	1 week	--	Mand. Ant. Mand.Max.Post
19			+		+		+	+	+	+	+	2 days	102	Mand. Ant.
20	+		+		+		+	+	+	+	--	3 days	99.6	Generalized
21	+-	+	+			+	+	+	+	+	+	2 weeks	99.5	Mand. Ant. Max. Ant.
22		+	+	+			+	+	+	+	+-	4 days	--	Max. Ant.
23	+		+	+			+	+	+	-	+	1 day	100.1	Mand. Ant.
24	+		+		+		+	+	+	+	+	2 days	100.1	Max. Ant. Entire Mouth
25	+					+	+	+	+	+	+	1 day	100.0	Mand. Ant. Mand. Post
26	+		+			+	+	+	+	+	+	1 day	99	Mand. Ant. Mand. Post
27	+		+			+	+	+	+	+	+	1 day	100	Max. Ant.
29		+	+		+		+	+	+	+	+	2 days recurrent	--	Mand. Ant. Max. Post
30	+		+			+	+	+	+	+	+	1 day	99.2	Entire Mouth
31		+	+			+	+	+	+	+	-	6 months recurrent	99.0	Entire Mouth
32	+		+			+	+	+	+	-	+	1-2 days	--	Max. Ant.

[a] no
[b] slight
[c] normal

10. When asked if the gums of the patients were painful prior to coming to the clinic:

71% painful 5 days or more
13% painful 4 days
8% painful 3 days
8% painful 2 days

11. When asked if their gums had ever been painful before:

61% stated never
21% stated once
11% stated more than three times
7% stated twice

12. When asked when was their last episode of painful gums: mean 30 months with a range of 6 months to 4 years ago.

13. When asked what they were doing at the time of the last episode:

They stated they were either working or that this was the first episode.

14. When asked if their gums bleed when they brushed their teeth:

67% said yes; 33% said no.

15. When asked if their gums every bled by themselves - without brushing:

63% said no; 37% said yes

16. When asked how long their gums had been bleeding prior to their coming to the clinic:

10% one day
15% two days
10% three days
10% four days
55% five days or more

17. When asked if this spontaneous bleeding ever happened before:

71% stated never
5% stated once
24% stated more than 3 times

18. The last spontaneous bleeding episode of the patients was: mean 6 months with a range of one-12 months

19. When asked what they were doing during their last episode:

Working, unemployed or in school

20. When asked if they smoke:

81% did smoke; 19% did not

21. When asked how much they smoked:

73%	0-1 pack/day
18%	1-2 packs/day
18%	over 2 packs/day

22. When asked how much sleep they had per night the week prior to visiting the clinic:

11%	0-4 hours
33%	4-6 hours
41%	6-8 hours
15%	over 8 hours

23. When asked how much sleep they had per night this last month:

—	0-4 hours
15%	4-6 hours
56%	6-8 hours
29%	over 8 hours

24. When asked if they sleep restfully when they do sleep:

74% stated yes; 26% said no

25. When asked if they were working at a job for which they were best suited:

60% stated yes; 40% stated no

26. When asked how many hours per day they were at their job:

10%	0-2 hours
15%	2-4 hours
—	4-6 hours
30%	6-8 hours
35%	8-10 hours
10%	over 10 hours

27. When asked how many days per week they were at their job:

9%	0-2 days
5%	3 days
14%	4 days
64%	5 days
4%	6 days
4%	7 days

28. When asked how many meals they ate each day:

11%	1 meal
50%	2 meals
28%	3 meals
11%	more than 3 meals

29. When asked how many meals they ate in a "fast food" establishment: Of the 50% which ate in fast food restaurants:

54% ate one meal
31% ate two meals
15% ate three meals

30. When asked if they felt they ate well:

71% stated yes; 29% stated no

31. When asked if they have time to brush their time:

96% answered yes; 4% no

32. When asked how often they brush their teeth each day:

4%	less than once per day
37%	once per day
42%	twice per day
12%	three times per day
5%	never

33. When asked how often they floss each day:

11%	less than once per day
18%	once per day
11%	twice per day
3%	three times per day
57%	never

34. When asked if the patients felt they had enough time to accomplish their responsibilities:

65% stated yes; 55% no

35. When asked if they enjoyed their current living situation:

69% stated yes; 31% no

36. When asked if they enjoyed their present job:

61% stated yes; 39% no

37. When asked if the present condition of their mouth affected their work:

52% stated yes; 48% no

38. Race

18 caucasion; 13 black

CULTURING SUBGINGIVAL PLAQUE AND TISSUE SURFACE SCRAPINGS FOR MICROORGANISMS

At the present time samples have been taken from 30 ANUG patients. Seven of these samples were used in our initial studies in developing the cultural and identification procedures. Also attempts were made initially to isolate Fusobacterium nucleatum and Bacteroides strains from ANUG patients for use in the serologic studies to be presented. Plaque samples from the patients have revealed the isolation of approximately 8-12 different colony types from each patient. The procedure for culturing and identification of the samples follows:

Media

The following media were used: Crystal Violet-Erythromycin (CVE) agar; MM10 agar; and Trypticase Soy Agar (TSA) with hemin and menadione (TSAHK). CVE agar (pH 7.2 contains in g/l the following: trypticase (10.0), yeast extract (5.0), NaCl (5.0), Tryptophan (0.2), agar (15.0) and crystal violet (0.001), erythromycin (0.004), and defibrinated sheep blood (50 ml) which were added after autoclaving. MM10 contained/liter: H_20 (890 ml), 37.5 ml of 0.6% K_2HPO_4, 37.5 ml of a salt solution (NaCl), 1.2 g; $NH_4{}_2SO_4$, 1.2 g; KH_2PO_4, 0.6 g, Mg_2SO_4, 0.25 g and 100 ml H_20, bacto-agar (15.0 g), trypticase (2.0 g), yeast extract (0.5 g), sucrose (30.0 g), KNO_3(0.25 g), 0.05% hemin, (2.0 ml) and cysteine (0.12 g), 8% Na_2CO_3 (5 ml), DL-dithio-threitol (0.1 mg) and sheep blood (20 ml) which were added after autoclaving. TSA contained 40 g/l trypticase soy agar and for the preparation of TSAHK 10 ml of 0.5 mg/ml hemin in 0.1 N NaOH, 1 ml of 0.1% menadione in 95% ETOH and 50 ml of defibrinated sheep blood were added.

Microbiological Culturing of ANUG Lesion

Samples of subgingival plaque were collected using a curette under nitrogen gas and immediately placed into 1 ml of reduced transort fluid (RTF) containing an Eh indicator (58). The vial was placed into a Coy anaerobic chamber, vortexed for 30 seconds and the contents of the vial diluted in serial ten-fold dilutions with RTF. Each dilution (0.1 ml) was spread plated onto 3 reduced MM10 agar plates. Also 0.1 ml of the 10^{-1} dilution was

spread plated onto CVE agar plates and TSAHK. The plates were allowed to incubate for 3 days, after which time they were inspected and the dilution showing 30-200 colonies/MM10 plate was used for quantitation of the viable count. The average of the colony counts for the 3 plates at that dilution was multiplied by the reciprocal of the dilution times 10. The number of colony types on each plate were observed and the average number of each colony type on the plates determined. The % of each colony type in relation to the total viable count was calculated. Each colony type was Gram stained and subcultured for isolation onto MM10 plates and incubated anaerobically for 2 days. Also each colony type was subcultured onto another MM10 plate and incubated aerobically for oxygen tolerance. Each isolate from MM10 was subcultured to BHI broth and allowed to grow 48 h and then placed into litmus milk and frozen. Anaerobes were identified by the API 20 anaerobe system. Facultative anaerobes and gram positive cocci were identified by the API 20S system. Several streptococci were identified by their colonial morphology on MM10, for example, S. sanguis (transparent, shiny, immovable, rigid colonies), S. salivarius (large, transparent, sticky, mucoid colonies) and S. mutans (colorless, granular, indented, small colonies). Black pigmented colonies were taken from the TSAHK and subcultured for isolation on TSAHK and then identified via the use of the API 20A system. F. nucleatum colonies isolated from the CVE agar were also identified with the API 20A system. Sonicated suspensions of the isolates were also identified via reaction by immunodiffusion with rabbit antiserum to prototype strains.

RESULTS

The results of individual cultural studies on ten of the ANUG patients are presented in Tables 2-11. The results of the cultural studies of twenty of the patients have been combined and presented in Table 2. As can be observed in Table 12, to date the Gram - rods comprised the highest percentage of the total organisms isolated. Of this group B. gingivalis and F. nucleatum were the most frequently isolated with B. intermedius, other Fusobacterium sp. and Vibrio sp. also being identified. The Gram + cocci comprised 15.5% of all of the isolates, Streptococcus and Staphylococcus sp., Pseudomonas micros,

and Peptostreptococcus sp. being isolated. A smaller number of Gram + rods than expected was observed. These included isolates of C. beijerinckie, L. fermentum and A. israelii. V. parvula was the only identified Gram - coccus isolated from these patients.

ANUG I

Table 2

White Male - Total counts on MM10 at 10^{-4}: 1. 90, 2. 96 — Avg. Total Viable Count (using 0.1 ml) 93×10^3

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Strep. intermedius	42×10^5	45.2		x		x
Strep. morbillorum	19×10^5	20.4		x		x
Bacteroides gingivalis	2×10^5	2.2		x	x	x
Fuso. nucleatum	2×10^5	2.2		x	x	x
Strep. sanguis	7×10^5	7.5		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Med. brown with dk. ctr.	Gm variable cocci	(+)	1×10^5	1.1	20S - all neg.
Smooth, flat, shiny, greenish-gray	Gm (+) cocci chains	(+)	1×10^5	1.1	ND
Creamy-white, raised with Beta hemolysis	Gm (-) rods	(-)	7×10^5	7.9	20A - $S^-E^-C^+$
Tiny, beige, smooth, raised with reg. edge	Gm (-) cocci	(+)	12×10^5	12.9	N/A

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

ANUG II

Table 3

Black Female - Total counts on MM10 at 10^{-3}:
1. 152
2. 164

Avg. Total Viable Count (using 0.1 ml)
158×10^4

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Peptostrep. prevotii	92×10^4	58.2		x	x	
Veillonella parvula	8.5×10^4	5.4		x	x	
Peptostrep.	2×10^4	1.3		x	x	
Bacteroides gingivalis	1×10^4	0.7		x	x	x
Strep. sanguis	1×10^4	0.7		x	x	x
Fusobacterium spp.	23×10^4	14.61		x	x	x

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Mucoid, red & white, smooth round, shiny	Small Gm (-) cocci	(-)	1×10^4	0.7	20A - $S^+E^-C^-$ (Lac, Sac)
Flat, round, with dark brown center, white rim	Gm (-) coccobacilli	(-)	4×10^4	2.6	20A - $S^-E^-C^-$
Green, raised, irreg. edge	Gm (+) sequented rods	(-)	0.5×10^4	0.4	20A - $S^-E^+C^-$ (Ind, Esc)
Small, pink, irreg. edge, raised, rough surface	Branching, grainy Gm (+) rods	(-)	3.5×10^4	2.2	20A - $S^-E^-C^-$
Dark brown, irreg. edge, raised surface	Gm (+) diptheroid-like rods	(+)	10×10^4	6.3	N/A
Shiny, smooth, round, irreg. edge	Shant, plump Gm (-) coccobacilli	(-)	1×10^4	0.7	20A- $S^-E^-C^-$

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 4

ANUG III

White Male - Total counts on MM10 at 10^{-3}:

1. 231
2. 274
3. 164

Avg. Total Viable Count (using 0.1 ml) 223×10^4

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
Fusobacterium nucleatum	33×10^4	14.8		x	x	
Veillonella parvula	4.3×10^4	1.9		x	x	
Strep. morbillorum	0.3×10^4	0.1		x	x	
Strep. sanguis	0.7×10^4	0.3		x	x	
Veillonella parvula	1.3×10^4	0.6		x	x	

Non-identified on MM10: classified according to G m reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Purple, raised center, pink rim, shiny, irreg. edge	Gm variable coccobacilli	+	4×10^4	1.8	20S - all neg
Dark red with pink rim, irreg. edge	Gm (+) rods short, plump	+	13.3×10^4	6.0	N/A
White, shiny, raised center	Short, plump Gm (-) rods	+	0.3×10^4	0.1	N/A
Small, dark, reddish-brown, convex	Long Gm (-) rods in short chains	-	16×10^4	7.2	N/A
Tiny, brown, smooth	Small Gm (-) rods	-	89×10^4	39.9	20A - $S^+E^-C^-$ (Lac, Sac, Mal, Rha)

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 5

ANUG XIV
22 YOBM - Total counts on MM10 at 10^{-4}:
1. 135
2. 99
3. 100

Avg. Total Viable Count (using 0.1 ml)
111.3×10^5/ml

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Bacteroides gingivalis	6.0×10^5	5.4		x	x	x
Fusobacterium nucleatum	5.0×10^5	4.5		x	x	x
Clostridium beijerinckii	0.6×10^5	0.6		x	x	
Streptpcoccus sanguis	1.0×10^5	0.9		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
White, raised, shiny smooth with regular edge	Gm (-) cocci in and chains	(+)	3×10^5	2.7	API 20S - all neg.
Raised, shiny mucoid smooth with regular edges	Gm (+) cocci in clusters	(+)	11×10^5	9.9	API 20S - all neg.
Black-pigmented raised with irrreg. edge. Smooth; lighter brown periphery.	Gm (-) cocco bacillus	(+)	6×10^5	5.4	N/A
Light brown smooth center with rough dark brown periphery	Gm (-) cocci	(+)	2.3×10^5	2.1	API 20S - all neg.
Brown, rough, flat colony with irreg. edge. Spreader	Gm (-) rods	(+)	1×10^5	0.9	N/A
White, smooth, mucoid colony with reg. edges, center raised to peak	Gm (-) long fusiform rods	(+)	2×10^5	1.8	N/A

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 6

ANUG XV
White Female - Age 42

Total counts on MM10 at 10^{-4}:
1. 60
2. 89
3. 99

Avg. Total Viable Count (using 0.1 ml)
82.6×10^5

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
Ps. micros	2×10^5	2.42		x	x	x
F. nucleatum	12.5×10^5	1.51		x	x	x
S. sanguis	1×10^5	1.21			x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Raised, shiny, brown light brown periphery, reg. edge	Gm (+) rods w/terminal spores	(+)	12×10^5	14.5	NI
white, raised, shiny, peaked center, reg. edge	Gm (+) cocci	(+)	3×10^5	3.63	20S - all neg.
White, dull, raised, center peak, irreg. edge	Gm (+) cocci	(+)	3×10^5	3.63	20S - (+) Leu
dark brown, pinpoint, raised, reg. edge	Gm (-) short blunt rods	(-)	13×10^5	15.7	20A - $S^+E^-C^-$ (Ind, Glu, Sac, Gel)
small, creamy, irreg. edge, light brown center peak, raised	Gm variable segmented branching rod	(+)	3.5×10^5	4.2	N/A
small, white, raised, reg. edge, shiny	Gm (-) cocci	(-)	7.3×10^5	8.8	20A - $S^+E^-C^-$ (Glu, Lac, Sac, Mal, Gly, Cel, Raf, Sor, Trc)

Table 6 con't.

On TSAHK

Organism at 10^{-3}
NI — 1 Black pigmenting colony — API 20A - $S^+E^-C^-$ (Glu, Lac, Sac, Mal)

On CVE at 10^{-3}

Organism	GS	COL	API	SEL MED
F. nucleatum	x	x	x	x
F. nucleatum	x	x	x	x
F. nucleatum	x	x	x	x

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 7

ANUG XVII
White Female - 12 years

Total counts on MM10 at 10^{-5}:
1. 75
2. 72
3. 78

Avg. Total Viable Count (using 0.1 ml)
75×10^6

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
F. nucleatum	4×10^6	5.3		x	x	x
S. sanguis	1×10^6	1.3			x	
Ps. anaerobotus or micros	$.5 \times 10^6$	.67		x	x	x

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
White, raised, smooth, shiny, reg. edge, large	Gm (-) thin fusiform rods	-	2×10^6	2.7	20A - $S^+E^-C^-$, Glu Man, Lac, Sac, Mal, Raf, Sor
Small, raised, tan, reg. edge	Gm (-) rods, med. length, thick, chains, curved	-	29×10^6	38.7	20A - $S^+E^-C^-$ - Glu, Man, Lac, Sac, Mal
Brown, raised, rough, irreg. edge, raised clear center	Gm (+) cocci	+	1×10^6	1.3	20S - all neg.
Brown, spreader, irreg. edge	Gm (-) rods, large thick, chains, w/ segmentation & single	-	1×10^6	1.3	20A - $S^+E^-C^-$ Glu, Lac, Sac, Mal, Sal, Cel, Mne, Raf, Tre
Brown, clear edge, raised dark center, reg. edge	Gm (-) rods, large, thick, chains, w/ segmentation & single	-	1×10^6	1.3	20A - $S^+E^-C^-$, Glu, Man, Lac, Sac, Mal, Mne, Raf, Sor, Tre
Small, white, reg. edge, raised	V. small Gm (-) rods, comma shaped	-	3×10^6	4	20A - $S^+E^-C^-$ - Glu, Man, Lac, Sac, Mal

Table 7 con't.

On TSAHK	Avg. Viable Count	% of Total Black-Pigmenting Organisms	ID			
			GS	COL	API	SEL MED
Bacteroides gingivalis	2×10^5		x	x	x	
On CVE						
F. nucleatum	7×10^5		x	x	x	

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

ANUG IX

Table 8

Total counts on MM10 at 10^{-4}:
1. 102
2. 61
3. 60

Avg. Total Viable Count (using 0.1 ml)
74.3×10^5

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
Clostridium beijerinckii	24×10^5	32.3		x	x	
Bacteroides gingivalis	10×10^5	13.5		x	x	x
Fusobacterium mortiferum	2.3×10^5	3.1		x	x	
Lactobacillus fermentum	5.0×10^5	6.7		x	x	
Peptostrep. prevotii	1.3×10^5	1.7		x	x	
Vibrio sp.	1×10^5	1.3		x	x	x

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Opaque, raised center, flat periph. center smooth, irreg. wrinkled edge	Gm (-) med. length rods	(+)	4.3×10^5	5.8	N/A
Smooth flat colonies with slightly raised center. Center dark brown, periph. lt. brown edge regular	Gm (-) coccobacilli	(+)	3.3×10^5	4.4	N/A
Rough, raised, opaque Irreg. edge. Immovable	Thin Gm (-) rods Branched & sequented	(+)	2×10^5	2.7	N/A
Flat white rough red. edge	Small, thin Gm (-) rods	(-)	1×10^5	1.4	Died
Pinpt. dark brown rough with irreg. edge	Gm (-) cocci, somewhat kidney-shaped mostly pairs	(+)	0.3×10^5	0.4	N/A

On CVE (selective for detection of presumptive F. nucleatum strains)
Two strains of F. nucleatum isolated & ID via GS, phase, API & growth (colonial morph)

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 9

ANUG XI
18 YO WM - Total counts on MM10 at 10^{-5}:
1. 77
2. 68
3. 38

Avg. Total Viable Count (using 0.1 ml)
61×10^6/ml

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
Streptococcus sanguis	0.3×10^6	0.5			x	x
Actinomyces israelii	3.3×10^6	5.4		x		

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Shiny, tan, slightly raised with clear rim and reg. edge	Gm (-) short blunt rods	(-)	8×10^6	13.1	20A - $S^+E^-C^-$ - Ind, Gly, Lac, Sac, Mal, Xyl, Raf, Rha
Brown with slightly white peaked center. Rough, immovable with irregular edge	Gm (-) shant blunt coccobacillus	(+)	4×10^6	6.6	20A - All neg.
Rough star-shaped with white center and brown periphery - Immovable with irreg. edge	Gm (-) cocci	(+)	1×10^6	1.6	20S - All neg.
Small, translucent, shiny smooth & slightly raised	Gm (-) cocci	(+)	5.7×10^6	9.3	20S - All neg.
Violet colored smooth slightly raised rigid with regular edge	Gm (-) long thin rod	(-)	0.3×10^6	0.5	Died

Table 9 con't.

Rough, slightly raised and brown with scalloped edge	Gm (-) cocci	(+)	2×10^6	3.3	20S - All neg.
Raised, translucent center brown periphery. Center smooth; periph rough. Spreader	Large Gm (-) rod	(+)	0.3×10^6	0.5	N/A

On TSAHK (selective for black-pigmenting Bacteroides)

One strain of B. mel. ss intermedius was isolated & ID via GS, API 20A, colonial morph (pigment)

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 10

ANUG XII
19 YO BF - Total counts on MM10 at 10^{-4}:
1. 35
2. 34

Avg. Total Viable Count (using 0.1 ml)
34.5×10^5/ml

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
Bacteroides gingivalis	12×10^5	34.8		x	x	x
Lactobacillus fermentum	7×10^5	20.3		x	x	
Fusobacterium varium	2.5×10^5	7.2		x	x	
Fusobacterium mortiferum	2×10^5	5.8		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Beige mucoid with reg. edge	Thick Gm (-) rods med. length, blunt ends	(-)	8.5×10^5	24.6	20A - $S^+E^-C^-$
Dull fried egg appearance colony with dark brown center and light brown periphery	Branching, long thin segmented Gm (-) rods	(+)	4.5×10^5	13.1	N/A

On TSAHK (selective for black-pigmenting Bacteroides)

Two strains of Bacteroides gingivalis isolated and ID by GS, API 20A, colonial morph.

On CVE (selective for detection of presumptive F. nucleatum strains)

No F. nucleatum detected

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 11

ANUG XIX
28 YO WM - Total counts on MM10 at 10^{-3}: Avg. Total Viable Count (using 0.1 ml)
JP superimposed 1. 43 39×10^4/ml
ANUG 2. 32
3. 42

On MM10 (nonselective) medium: ID

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	COL	API	SEL MED
B. intermedius	15.7×10^4	40.3		x	x	x
B. gingivalis	14.7×10^4	37.7		x	x	x
F. necrophorum	2.0×10^4	5.1		x	x	
S. constellatus	0.7×10^4	1.8		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Variable Cnt./ml	% Total Variable Cnt.	API 20A/20S
Small, creamy, raised smooth with regular edges	Gm (+) branched rods long & thin & exhibiting segmentation	(-)	3.7×10^4	9.5	20A - S⁻E⁻C⁻
Large, dark brown, dull, flat with regular edge	Gm (+) cocci in chains, clusters	(+)	0.7×10^4	1.8	20S - alpha hemolytic
Small, light brown center dark brown edge, flat, dull with irreg. edge	Gm (+) cocci in chains, clusters and pairs	(+)	0.3×10^4	0.8	20S - alpha hemolytic ARL - (+) Rest - (-)

On TSAHK (selective for black-pigmenting Bacteroides)

Organism	Avg. Viable Count/ml	% Total Viable Count of Black-Pigmenting Org's
B. intermedius	21.4×10^4	95.5
B. gingivalis	1.0×10^4	4.5

On CVE (selective for detection pf presumptive F. nucleatum strains)

No F. nucleatum detected

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 12

Levels of Suspected Odontopathic Organisms in Predominant Cultivable Flora of Plaque Taken from ANUG Sites of 20 Patients

Species	Total Avg. Count	% of Total Organisms
Gram + rods	1.17×10^7	2.66
Clostridium beijerinckie	2.46×10^6	.56
Lactobacillus fermentum	1.2×10^6	.27
Actinomyces israelii	3.3×10^6	.75
Unidentified	1.17×10^6	.26
$S^-E^-C^-$	7.2×10^4	.02
$S^+E^-C^-$	1.1×10^6	.25
$S^-E^+C^-$	2.0×10^3	.001
Facultative (unidentified) all nonhemolytic & ARL^-	3.54×10^6	.80
Gram + cocci	6.83×10^7	15.5
Facultative (identified)		
Streptococcus sanguis	4.7×10^6	1.17
Streptococcus sp.	5.04×10^6	1.15
Staphlococcus sp.	5.0×10^6	1.14
Facultative (unidentified)	4.15×10^7	9.43
2 hemolytic, ARL^+	3.14×10^7	7.13
2 hemolytic, ARL^-	7×10^4	.002
Beta hemolytic, ARL^-	2.5×10^6	.57
Nonhemolytic, ARL^+	2.3×10^6	.52
Nonhemolytic, ARL^-	5.17×10^6	1.18
Anaerobic (identified)		
Pseudomonas micros	7.0×10^5	.16
Peptostreptococcus species	1.2×10^6	.27
Streptococcus sp.	9.12×10^6	2.07
Anaerobes (unidentified)	9.2×10^5	.21
$S^+E^-C^-$	9.0×10^5	2.05
died	6.0×10^5	

Species	Total Avg. Count	% of Total Organisms
Gram - rods	3.44×10^8	78.2
Bacteroides intermedius	2.57×10^5	.06
Bacteroides gingivalis	3.43×10^7	7.80
Fusobacterium nucleatum	1.5×10^7	3.41
Fusobacterium sp.	4.93×10^6	1.12
Vibrio sp.	2.5×10^5	.06
Anaerobes (unidentified)	1.04×10^8	23.6
$S^+E^-C^-$	4.95×10^7	11.25
$S^-E^-C^-$	6.05×10^6	1.38
$S^-E^-C^+$	7×10^5	.16
Not tested	16×10^4	10.93
Died	4.81×10^7	10.93
Facultative all nonhemolytic and ARL^-	1.85×10^8	42.0
Gram - cocci	1.55×10^7	3.52
Veillonella parvula	3.3×10^6	.75
Anaerobes (unidentified)	1.15×10^6	.26
$S^+E^-C^-$	9.95×10^5	.22
Died	1.5×10^5	.034
Facultative all nonhmolytic & ARL^-	1.10×10^7	2.5

Hemagglutination Activity of ANUG Isolates

Oral strains of Fusobacterium nucleatum and Bacteroides gingivalis have been shown to attach to and cause hemagglutination (HA) of human and sheep red blood cells (59). This activity may be involved in the colonization and pathogenic mechanisms of these organisms in the gingival crevice (60). Studies were undertaken to see if Fusobacterium nucleatum and Bacteroides gingivalis and intermedius isolates from ANUG patients demonstrated HA activity.

Materials and Methods

Cultures and cultural conditions. The strains of Fusobacterium and Bacteroides used can be seen in Tables 13 & 14. The fusobacteria were grown in a modified tryptone medium (59) and the Bacteroides strains in Brain Heart Infusion broth supplemented with 5 µg/ml hemin and 2 µg/ml menadione using the BBL anaerobe jar-Gas Pak system. After 24 to 72 h of growth, the organisms were harvested by centrifugation at 10,000 x g for 10 min and washed three times with 0.15 M NaCl. The organisms were routinely resuspended in 0.01 M phosphate buffer containing 0.15 M NaCl and 0.2% sodium azide (PBS) at a concentration of approximately 0.28 g/ml or in a 10% suspension (packed volume, after centrifugation at 800 x g for 15 min, diluted 1:10 in PBS).

HA test. A modification of the microtiter test described by Crawford et al. (61) was used for HA testing. Twenty five microliters of the whole-cell suspensions were serially diluted twofold with microdiluters in a microtiter tray. To this was added 25 µl of PBS followed by 25 µl of a 3 x PBS-washed 1.25% sheep red blood cell suspension. The microtiter tray was shaken on a micromixer for 1 min and incubated at 37° C for 30 min followed by incubation at room temperature 2 h before reading. HA was recorded as 1 to 4+, 4+ being a smooth blanket of RBC covering the bottom of the well. PBS instead of the HA preparation was used as a control for normal buttoning of the RBC.

Results

As can be seen in Tables 13 & 14 the F. nucleatum strains displayed a wide range of HA activity whereas the Bacteroides strains displayed little if any HA activity.

Table 13

Hemagglutination Assay of F. nucleatum Isolates

F. nucleatum[a] strain	Hemagglutination Titer
VIII A_{14}	1:2
XII_3	1:8
WAF	1:1024
XA_{12}	1:4
VIII A_{13}	1:1024
CD_3	1:16
Lai	1:128
VI A_2	1:8
$XVII_3$	1:2
$XiII_2$	1:64
VI A_1	1:64
XVI_2	1:2
CD_2	1:4
Smoot	1:8
4355	1:1024
10197	1:64
10953	1:4
DS_1	1:1024
MR_3	1:8

[a] 5% whole cell suspensions
[b] diltuion displaying at least 2+ HA

Table 14

Hemagglutination Assay of Bacteroides Isolates

Bacteroides[a] strains	Hemagglutination Titer
XII_8 B. gingivalis	1:2
CS44 B. gingivalis	-
CS43 B. gingivalis	-
CS41 B. gingivalis	1:2
XV_6 B. gingivalis	-
XIX_9 B. intermedius	1:2
25261 B. intermedius	-
382 B. fragilis	-
25285 B. fragilis	-
MH 678 B. asaccharolyticus	-
687 B. asaccharolyticus	-
B. ovatus	-

[a] 5% whole cell suspensions
[b] dilution displaying at least 2+ HA

Serologic Characterization of Fusobacterium nucleatum ANUG Isolates with Other F. nucleatum Strains

Introduction

A study was undertaken to compare the hemagglutination activity and reaction of human sera and rabbit anti-F. nucleatum sera with F. nucleatum isolates obtained from humans demonstrating clinically healthy gingiva and various gingival and periodontal disease states. Additional isolates were obtained from dogs and from a Macaca mulatta monkey demonstrating spontaneous chronic periodontitis. The serologic reactions of the F. nucleatum isolates were compared with selected reference strains of F. nucleatum.

Materials and Methods

Cultures and cultural conditions.

F. nucleatum clinical isolates were obtained from the following sources: 1) human adults virtually free of periodontal diseases, 2) human children free of periodontal diseases, 3) human adults with diagnosed chronic periodontitis, 4) human adults with diagnosed acute necrotizing ulcerative gingivitis (ANUG), 5) human adults with diagnosed juvenile periodontitis, 6) two mature dogs, one mixed breed and one golden retriever, with clinical signs of early chronic periodontitis and 7) one Macaca mulatta monkey with spontaneous advanced chronic periodontitis (Table 15). Three typed strains of F. nucleatum (ATCC 10953 and VPI 4355 and 10197) were also utilized. All clinical isolates were obtained from subgingival plaque samples using a sterile curette (MC 17/18 - Hu Friedy) and immediately streaked onto CVE agar (62), a selective medium for the isolation of F. nucleatum. All samples were incubated at 37° C anaerobically using the BBL anaerobic jar-Gas Pak system (BBL, Cockeysville, Md.) or a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Michigan) with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. After four days of incubation, isolates were identified by colonial morphology (62) and described as type I, a 2 mm transparent smooth blue colony having an entire edge with a dark blue center or type II, a 1 mm to 2

Table 15

Source of F. nucleatum clinical isolates

Clinical Description	Number of Subjects	Number of Isolates
Human Children free from disease	2	3
Human Adults free from disease	6	7
Human patients with chronic periodontitis	3	3
Human patients with ANUG	5	5
Human patients with juvenile periodontitis	3	6
Canine	2	8
Macaca mulatta	1	11
TOTAL	22	43

mm transparent round or irregular blue colony with a speckled appearance. Selected colonies were streaked for isolation on blood agar (BBL) and incubation continued for 48 hr. Each isolate was observed by phase contrast microscopy and gram stained to verify the typical morphology of Fusobacterium. Only isolates which demonstrated Gram negative staining with the morphology of long rods with pointed ends and failing to grow aerobically on blood agar were selected for further study. Biochemical reactions were determined using the API 20A system (Analytab Products, Plainville, N. Y.). Isolates were then transferred to a modified tryptone medium (59) and incubated as before. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4° C, washed three times in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2 and resuspended in PBS to a 10% suspension (1:10 dilution of packed whole cells after centrifugation at 2,000 x g for 10 min).

Whole cell suspensions of these isolates were sonicated using 8 bursts of 30 sec each in a dry ice-alcohol bath with a Heat Systems Sonicator (Plainville, N. Y.) at a microtip setting of 7. Greater than 95% of the cells were lysed as observed by phase contract microscopy. This was designated as the sonicated preparation (SP).

Double diffusion in agar.

SP of selected isolates and typed strains of Fusobacterium nucleatum were reacted by double diffusion (63) in 1% agarose in 0.01 M PBS, pH 7.2 with undiluted rabbit anti-F. nucleatum 10953 or 10197 serum (59). Reactions were incubated at 4° C in a humidor and were observed after 24 hr. Protein content of SP as determined by Lowry et al. (64) was aproximately 400 µg/ml).

Serological evaluation by ELISA.

Selected isolates were reacted by an enzyme-linked immunosorbent assay (ELISA) with the following sera: 1) rabbit anti-F. nucleatum 10953 or 10197 serum, 2) normal rabbit serum, 3) human sera previously shown to be reactive to 10953, 10197 and 4355 and 4) serum obtained from the Macaca mulatta demonstrating spontaneous chronic

periodontitis. A modification of the enzyme-linked immunosorbent assay (ELISA) was utilized (65). Two hundred microliters of a 1:10 dilution of the previously described whole cell suspensions of F. nucleatum in a 60 mM carbonate buffer, pH 9.6, were added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) and incubated at 37° C for 3 hr followed by refrigeration overnight. Peripheral rows were not utilized. The plate was washed five times with 0.01 M PBS containing 1% BSA and 0.05% tween 20. This was followed by a 3 hr incubation at 37° C with 200 µl of a 1% BSA solution in 60 mM carbonate buffer in order to assure complete coverage of all binding sites in each well. The plate was then washed five times with PBS/tween 20 with 1% BSA. One hundred microliters of serial two fold dilutions in 0.01 M PBS with 0.05% tween 20, pH 7.2, of the sera were added and incubated at 37° C for 30 min. The plates were washed as before and 100 µl of a 1:200 dilution in PBS of peroxidase labeled IgG fraction of goat anti-rabbit γ , α and μ heavy chain serum or peroxidase labeled goat anti-Rhesus gamma/globulin serum (Cappel Laboratories, Inc., Cochranville, Pa.) were added and the plate incubated again at 37° C for 30 min. After again washing the plates five times, 100 µl of the enzyme substrate (1 ml of 1% w/v O-phenylene-diamine in methanol plus 99 ml distilled H_20 and 0.1 ml 3% H_20_2) were added. The plates were incubated in the dark for 30 min at room temperature, the reaction was stopped by adding 20 µl of 8N H_2SO_4 and reactions were determined colormetrically at 490 nm with a Microelisa Reader (Dynatech Laboratories, Inc.). To determine the optimal concentration of antigen and peroxidase labeled goat antiserum, a dual titration of doubling dilutions of antigen (0.25% to 10% whole cell suspensions) against serial 1:5 fold dilutions of conjugate was performed. Analysis of the protein content of the 1% whole cell suspensions (1:10 dilution of the 10% suspension) was determined to be 400 µg/ml by the technique described by Lowry et al. (64).

Hemagglutination (HA)

The HA assay was performed by the method of Falkler and Hawley (60). Briefly,

serial two-fold dilutions of 50 µl of 10% whole cell suspensions of selected isolates of F. nucleatum were made in microtitration multi-well plates (Linbro Scientific, Inc., Hamden, Conn.) with 0.01 M PBS, pH 7.2. This was followed by the addition of 25 µl of PBS, pH 7.2 and 25 µl of a 1.25% suspension of sheep red blood cells (RBCs). Plates were mixed for 20 sec on a Micro-Shaker (Cooke Laboratory Products, Alexandria, Va.) then incubated for 30 min at 37° C followed by 1 hr at 4° C. Titers were determined visually as the reciprocal of the highest dilution demonstrating a 2+ hemagglutination. Inhibition of HA was determined as above except for the addition of 25 µl of 50 mM of D-galactose in place of PBS and incubation for 30 min at 37° C prior to adding the sheep RBCs.

Results

Isolation of F. nucleatum

F. nucleatum isolates were obtained on CVE agar plates from all plaque samples. The human isolates demonstrated both type I and type II colonial morphology as did isolates from the Macaca mulatta monkey. All canine isolates showed only type I colonial morphology. All isolates regardless of colony type showed identical results with the API 20A system (a positive indole response with all other reactions negative).

Serological evaluation by immunodiffusion.

SP of 10953, 10197 and 4355 were reacted with rabbit anti-F. nucleatum 10953 serum by double diffusion in agar. Several precipitin lines including lines of identity were observed for all typed strains. SP of sixteen separate isolates from various human oral disease states and the three typed strains of F. nucleatum were also reacted with rabbit anti-F. nucleatum 10953 serum. Lines of identity were evident between all sixteen clinical isolates of F. nucleatum and the three typed strains. The eleven Macaca mulatta monkey isolates, eight canine isolates, three human isolates and strain 10953 were also reacted with the rabbit anti-F. nucleatum 10953 serum. Of all nineteen of the animal isolates tested, only two of the Macaca mulatta strains shared lines of identity with the human isolates and 10953.

Eleven human isolates of F. nucleatum were reacted by ELISA with rabbit anti-F. nucleatum 10197 or 10953 serum and with normal rabbit serum. As can be observed in Table 16, similar titers for all isolates were obtained with both hyperimmune sera which displayed substantially higher titers than those observed with normal rabbit serum.

Twenty-three human clinical isolates of F. nucleatum were reacted by ELISA with a human serum previously shown to be reactive with F. nucleatum. The titers that were obtained are shown in Table 17. Irregardless of the source of the F. nucleatum isolates, a similar range of antibody activity was detected with the human serum.

The results obtained when ten Macaca mulatta isolates, eight canine isolates, four human isolates and 10953 were reacted with 1) human sera previously shown to be reactive with F. nucleatum, 2) serum from the Macaca mulatta with spontaneous periodontal disease and rabbit anti-F. nucleatum can be seen in Table 18. All sera showed antibody reactivity to the human (including 10953) and Macaca mulatta strains but not to the canine isolates.

Hemagglutination (HA)

Eleven Macaca mulatta isolates, eight canine isolates, three human isolates and srain 10953 were tested for HA activity and with the addition of 50 mM D-galactose, for inhibition of HA. All canine isolates consistently failed to shown any HA of sheep RBCs (Table 19). Some of the Macaca mulatta isolates and all of the human isolates showed varying degrees of HA activity. All isolates which demonstrated HA activity also showed a decrease in HA when preincubated with 50 mM D-galactose prior to HA testing.

Table 16

Elisa titers obtained with human isolates of *F. nucleatum* and anti-*F. nucleatum* and pre-immune sera.

Patient Classification	Sera: Rabbit anti-*F. nucleatum* T0197 mean titer	Rabbit anti-*F. nucleatum* T0953 mean titer	Normal rabbit mean titer
Normal adult (6)[a]	6[b]	6	3
Patients with acute necrotizing Ulcerative Gingivitis (5)	6	6	3

[a] number of patients providing isolates of *F. nucleatum*

[b] highest dilution giving an OD reading of greater than 0.15 at 490 nm after transforming the results from geometric progressions to arithmetic progressions (1:16=1, 1:32=2, 1:64=3, etc.

Table 17

Elisa titers obtained with human isolates of

F. nucleatum and a human sera

Patient Classification		mean titer[b]
Normal adult	7[a]	5.7 (5-6)[c]
Normal child	3	6[d]
Acute necrotizing ulcerative gingivitis	5	5.6 (4-6)
Chronic periodontitis	2	7[c]
Juvenile periodontitis	5	6.8 (6-8)

[a] number of patients providing isolates of F. nucleatum and number of isolates tested

[b] after transforming the results from geometric progressions to arithmetic progressions (1:10=1, 1.20=2, 1:40=3, etc.)

[c] range of titers

[d] all titers were the same within this group

Table 18

Elisa titers obtained with clinical and typed strain isolates of F. nucleatum with selected sera.

F. nucleatum isolates		Human	Rabbit anti-Macaca mulatta	F. nucleatum 10953 Serum
Macaca mulatta	10[a]	2.9[b] (3-4)[c]	4.7 (4-5)	5.7 (5-6)
Canine	8	1	1	1
Human	4	5.5 (5-6)	4[d]	7
10593	1	6	4	8

[a] number of isolates from each source

[b] after transforming the results from geometric progressions to arithmetic progressions (1:10=1, 1:20=2, 1:40=3, etc.)

[c] range of titers

[d] all titers were the same

Table 19

Hemagglutination obtained from clinical isolates of F. nucleatum and 10953.

Isolates		Hemagglutination titers	Hemagglutination titers after galactose inhibition
Macaca mulatta	11	3.3[b] (1-8)[c]	.91 (1-1)
Canine	8	[d]	[d]
Human	3	6.3 (4-8)	4.3 (3-6)
Typed strain 10953	1	11[e]	9[e]

[a] number of isolates from each source

[b] highest dilution displaying at least a 2+ HA after transforming the results from geometric progressions to arithmetic progressions (1:2=1, 1:4=2, 1:8=3, etc.)

[c] range of titers

[d] hemagglutination was not observed at the lowest dilution used - 1:2

[e] all titers were the same

Reaction of Serum from ANUG Patients with Fusobacterium and Bacteroides Strains

In an attempt to detect antibody in the sera of ANUG patients with Bacteroides and Fusobacterium strains enzyme linked immunosorbent assays were established. Sera from ANUG patients and age and sex matched healthy individuals were reacted with Fusobacterium nucleatum and Bacteroides strains isolated from ANUG patients, chronic periodontitis patients, non-oral abscesses and healthy gingival sulci. Experiments were conducted to measure reactive isotypes of IgA, IgM and IgG and in other experiments IgG only

MATERIALS AND METHODS

Micro-Enzyme-Linked Immunosorbent Assay (ELISA)

Briefly, 200 l of a 1% Fusobacterium or Bacteroides whole cell suspensions (grown as previously described in HA methodology) in .06 M carbonate buffer, pH 9.6, were added to each well of a microtiter multi-well plate (outside rows of wells not used) and incubated at 37° C for 3 h followed by refrigeration overnight. The plate was washed three times with PBS containing 0.05% Tween 20 and 1% BSA. Then 200 µl of a 1% BSA solution in 0.06 M carbonate buffer, pH 9.6, were added to the wells and incubation allowed to proceed for 3 h at 37° C in order to tie up all non-antigen coated binding sites. The tray was then rinsed five times with PBS/Tween 20 with a 1% BSA. Serial two fold dilutions of the sera (50 µl) in PBS were added to each well and the plates incubated at 37° C for 30 min. The plates were washed as before and Fusobacterium and Bacteroides absorbed peroxidase labeled IgG fraction of goat anti-human γ, α or μ heavy chain serum (Miles Laboratories) diluted 1:200 in PBS, was added to each well (50 µl) and incubated for 1.5 h at room temperature. After again washing the plates, 50 µl of the enzyme substrate (1 ml of 1% w/v O-phenylene-diamine in methanol + 99 ml distilled H_2O_2) were added. After 1.5 h of incubation in darkness at room temperature the reaction was stopped by the addition of 8 N H_2SO_4 (25 µl) and the color was read at 490 nm using a Chromoscan spectrometer.

To determine the amount of Bacteroides and Fusobacterium (antigen) required to coat the wells of the microtiter plate and to determine a dilution at which the peroxidase labeled goat antisera should be used, a dual titration of doubling dilutions of antigen (0.25 to 10% whole cell suspensions) against serial 1:5 fold dilutions of conjugate was performed. An excess of reactive sera (1 in 100 dilution) was added to each well. A 1% suspension of Bacteroides and Fusobacterium strains and a 1:200 dilution of conjugate were found to be concentrations which would give an optimum yellow color and were the standard dilutions used for all subsequent experiments. All serologic tests were performed in duplicate and when more than a 0.15 O.D. difference was observed, testing of the serum was repeated. Controls included the reaction of sensitized wells with saline replacing human serum in the assay and reacting substract with conjugate to check on enzyme activity.

RESULTS

Five sera from ANUG patients and age matched sera from individuals with healthy mouths were reacted with 18 strains of F. nucleatum and 12 Bacteroides strains. The microorganisms represented ATCC and VPI strains, clinical isolates and isolates from ANUG lesions.

When the five sera were reacted with the 18 F. nucleatum isolates in an ELISA which would detect IgG, IgA and IgM it was observed that the sera displayed similar degrees of activity irregardless of the F. nucleatum strain used as the antigen source (Table 20). It thus appears all of the F. nucleatum strains tested displayed shared antigenic determinants. It was also observed that there were differences in the activity of the sera, some showing significant differences in the amount of antibody reactive with the F. nucleatum strains. When IgG was detected in the ANUG sera and age matched sera from individuals with healthy oral cavities (Table 21) it was observed that there were no differences in the mean antibody activity to the 18 strains in the two groups and in three of the five sera pairs, a slightly higher level of antibody was detected in the sera from the healthy individuals. Again it appeared that IgG was present in each sera to all of the organisms tested and that similar levels of antibody reactive with each organism was detected.

Although a somewhat higher level of antibody was found reactive with the Bacteroides strains, the data paralleled what was observed with the F. nucleatum testing. It appears that the B. gingivalis strains isolated from the ANUG patients share antigens with other B. gingivalis clinical isolates (Table 22) and that no differences were observed between the reactivities of the patient and age matched control sera (Table 23). It was of interest that the levels of antibody in both groups reactive to the non-oral B. fragilis and B. ovatus strains were lower than those observed for the oral strains (Tables 24 & 25).

Table 20

ELISA Measuring IgG, IgA & IgM

F. nucleatum strain	ANUG Patient Serum Source XII	XIV	XV	XVI	XIX
4355	528[a]	435	861	330	997
Lai	716	508	1169	463	954
10953	1028	725	1166	560	1223
CD_3	495	243	930	140	720
VIII A_{14}	506	271	581	207	681
X A_{12}	444	323	827	105	751
$XIII_2$	397	206	629	12	568
WAF	460	247	746	126	690
VI A_1	678	307	1215	162	872
$XVII_8$	396	610	1022	565	910
XVI_2	705	768	983	505	1059
DS_1	444	225	704	111	744
XII_3	520	366	839	199	1010
CD_2	429	346	836	66	721
MR_3	462	345	888	23e	738
VI A_2	571	433	1131	127	761
VIII A_{13}	289	220	574	33	612
10197	560	490	1029	214	860
Mean =	535	393	896	232	826
S. E. =	121	89	202	52	186

[a] optical density at 490 nm - average of duplicates

Significant difference (by S. D.) between 1) serum XV and XIV, XVI; 2) serum XIX and XIV, XVI

Table 21

ELISA Measuring IgG

F. nucleatum strain	XII	Control	XIV	Control	XV	Control	XVI	Control	XIX	Control
4355	725[a]	1383	--[b]	1645	1240	746	378	1999	1363	1311
Lai	587	410	905	1635	1373	1005	708	773	1216	762
10953	588	734	1153	1600	1086	1003	697	629	1488	798
CD 3	162	227	589	1072	634	662	399	453	1043	538
VIII A14	292	493	--	995	701	1013	423	707	1032	360
X A12	221	329	955	480	741	515	654	437	1122	480
XII 2	331	284	1481	392	392	650	450	539	1078	366
WAF	412	221	1104	547	827	545	788	616	1192	563
VI A	753	308	1072	732	1589	557	881	787	1035	990
XVII 8	811	636	1457	748	1091	830	1216	689	1399	554
XVI 2	592	760	1319	1053	942	1294	--	494	321	667
DS 1	680	351	492	939	795	1025	--	498	1063	559
XII 3	464	559	529	1048	1122	865	1083	614	1021	697
CD 2	296	308	383	946	774	600	425	560	1088	398
MR 3	509	966	486	1228	1210	568	843	427	1085	782
VI A2	497	879	--	1129	1534	755	792	619	1253	761
XVIII A8	682	234	478	1544	1012	801	746	932	995	389
10197	574	731	--	1375	811	776	1054	748	1054	320
Mean	509.8	545.2	689.1	1061.6	993.0	789.4	640.9	695.6	1082.1	627.5
S.E.	120.2	128.6	162.5	250.4	234.2	186.2	151.2	164.1	255.2	148.0

[a] optical density at 490 nm
[b] no activity

Table 22

ELISA Measuring IgG, IgA and IgM

B. gingivalis strain	ANUG Patient Serum Source					
	XII	XIV	XV	XVI	XIX	X/SD
XV 6	738[a]	574	1448	672	1501	98.6/449.6
XII 8	627	709	1009	738	982	823.0/167.7
CS41	723	510	1221	283	1614	870.2/541/4
CS43	1194	921	1590	609	1596	1182.0/928.5
CS44	1093	823	1705	582	1663	1161.2/503.4
Mean =	863.0	707.4	1394.6	586.8	1471.2	
S.D. =	239.5	170.1	281.1	187.4	279.7	

[a] optical density at 490 nm - average of duplicates

Table 23

ELISA Measuring IgG

B. gingivalis strain	XII	Control	XIV	Control	XV	Control	XVI	Control	XIX	Control	ANUG X/SD	Control X/SD
XV6	1349[a]	1730	1622	1223	1690	1736	1613	1745	1680	1496	1590.8/139.4	1586.0/228.2
XII8	406	489	--[b]	608	675	175	373	861	1055	399	501.8/391.7	334.8/244.0
CS41	1407	1802	311	1133	1390	1660	1235	1738	1620	406	1192.6/511.5	1347.8/589.3
CS43	1640	1538	1643	1670	1551	1697	1011	1805	1738	419	1515.6/290.3	1427.8/566.4
CS44	1675	1679	1551	--	1432	1660	588	1941	1392	710	1327.6/428.0	1198.0/817.0
Mean	1295.4	1447.6	1025.4	926.8	1347.6	1385.6	964.0	1618.0	1497.0	688.0		
S.D.	517.0	544.5	802.4	640.9	393.7	677.4	496.9	431.0	279.8	470.0		

[a] optical density at 490 nm
[b] no activity

Table 24

ELISA Measuring IgG, IGA and IgM

Bacteroides strain	ANUG Patient Serum Source XII	XIV	XV	XVI	XIX	X/D
B. asacch	1557[a]	1337	1614	1042	1470	1404.4/227.7
XIX_9 (B. intermedius)	566	385	1804	140	1582	895.4/747.8
B. intermedius	996	937	1555	722	1431	1128.2/351.0
B. mel CS1	1028	822	1614	1243	1580	1257.4/344.1
B. frag 382	1060	709	1200	432	819	844.0/300.8
B. frag	572	166	584	67	511	380.0/244/6
B. ovatus	462	315	740	109	968	518.8/340.4
Mean =	891.6	667.3	1301.6	536.4	1194.4	
S.E. =	336.4	251.8	490.9	202.4	450.5	

[a] optical density at 490 nm - average of duplicates

Table 25

ELISA Measuring IgG

Bacteroides strain	XII	Control	XIV	Control	XV	Control	XVI	Control	XIX	Control	ANUG X/SD	Control X/SD
B. asacch.	772	895	1320	1386	1545	1095	1179	684	1407	831	1244.6/295.8	978.2/471.6
XIX9 (B. intermedius)	899	1247	1296	972	1705	528	775	708	1654	1030	1265.8/424.2	897.0/281.8
B. intermedius	1538	1824	1643	1670	1744	1750	1574	1582	1677	1637	1635.2/81.9	1692.6/95.4
B. mel CSI	1609	1871	1506	1790	1683	1677	1763	1920	1736	1567	1659.4/104.0	1765.0/143.9
B. frag 382	892	606	673	1263	1470	1189	495	674	1050	751	882.5/424.0	896.6/306.2
B. frag	813	261	--[b]	700	499	385	332	271	858	398	500.4/355.1	403.0/177.6
B. ovatus	200	62	1630	--	532	751	883	529	1518	309	952.6/617.8	330.2/315.4
Mean	960.4	966.6	1152.6	1111.6	1311.1	1053.6	1000.1	909.7	1414.3	931.9		
S.E.	362.4	364.7	434.9	419.4	494.8	397.4	377.4	343.3	533.7	351.6		

[a] optical density at 490 nm
[b] no activity

This data suggests that:

(1) Fusobacterium as well as Bacteroides isolates from ANUG lesions each share antigenic determinants.

(2) A single serum sample taken during an ANUG episode when reacted with Fusobacterium or Bacteroides strains appears not to be useful in the diagnosis of the disease.

Studies on Spirochetes from ANUG Lesions

Attempts were made to determine the number of rods, cocci and spirochetes in the plaque samples and to cultivate spirochetes from these samples. Plaque debris and extracted teeth were studied by transmission and scanning electron microscopy respectively to evaluate the spirochetes involved. In order to characterize the spirochete isolates serological studies and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis was performed. The materials and methods and results of these studies to date follows.

MATERIALS AND METHODS

Dark-field microscopic counts

The RTF plaque samples used in the culture attempts were vortexed for 30 sec using a vortex-genie and then a drop of each plaque suspension was placed in the center of a Petroff-Hausser Counting Chamber which had been covered with a No. 1.5 cover glass. The counting chamber was then allowed to stand 15 min at room temperature and was subsequently examined under dark-field at a magnification of 500 X using a Leitz Dialux microscope. All bacteria observed in 80 squares of the counting chamber were counted and divided into 3 categories: (1) rods, (2) cocci, and (3) spirochetes respectively. All counts were completed within 24 hours of plaque collection.

Isolation of Spirochete Strains

Subgingival plaque samples were collected by a periodontist from clinic patients. These samples were placed in a tube containing prereduced Medium A Broth (described below) and transported to a Coy anaerobic chamber. This chamber was maintained at a temperature of 35° C and with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. The samples were treated as described by Leschine and Canale-Parola (66) with the plaque sample serially ten-fold diluted in 13 x 100 screw-capped tubes containing the NOS medium with 1-2 µg/ml of rifampin and 0.3% agar. The tubes were then incubated inside the chamber for 7-14 days at which time the resulting individual isolated spirochetal colonies were picked and examined by dark-field microscopy to

determine the presence of spirochetes. Positive colonies were streaked on the appropriate medium in the chamber to obtain isolated clones or rediluted as before in fresh NOS media. A portion of the sample was also placed on 25 mm-0.45 µm pore size Millipore filters on the surface of Medium A containing 1.0% agar. The spirochetes migrated through the filters and the larger contaminating bacteria remained on the filter surface. Spirochetes developed as a veil of growth and the leading edge of the growth was removed by taking a plug of agar with a Pasteur pipette and restreaking on solid medium within the anaerobic chamber to obtain isolated clones.

Spirochete Cultivation

The oral spirochete strains which we have require slightly different growth media. The composition of the media used follows:

Medium A

Component	Amount
Mycoplasma Broth (BBL)	21 g/l
Thiamine Pyrophosphate	10 µg/ml
Sterile Rabbit Serum	
Agar (Oxoid)	10.0 g/l
Heat inactivated (56° C for 30 min)	10% V/V
pH 7.4	

NOS Medium

NOS-Part A

Component	Amount
Heart infusion broth (Difco)	1.25 g
Trypticase (BBL)	1.0 g
Yeast Extract (Difco)	0.25 g
Sodium thioglycollate	0.05 g
L-cysteine-HCl	0.1 g

L-asparagine	0.025 g
Agar (Oxoid)	0.3 g
Distilled water	90 ml

Final pH 7.0

NOS-Part B

Component	Amount
2% $NaHCO_3$ (rifampin for isolation 20 µg/ml) filtered sterilized.	1.0 ml/9.0 ml Part A

VFA-TPP-Serum

1.5 ml 0.2% thiamine pyrophosphate

1.0 ml VFA soln

10 ml sterile, heat inactivated rabbit serum. 0.25 ml/9.0 ml Part A

Volatile Fatty Acid Solution (VFA)

500 l Valeric Acid

500 l Isovaleric Acid

500 l Isobutyric Acid

500 l DL-2-Methyl Butyric Acid

Scanning electron microscopy (SEM) of extracted teeth

Freshly extracted teeth were immediately immersed in 0.2 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde (GA) and fixed at 4° C for 16 to 24 h. Following the prefixation in GA, the specimens were washed 3 times with 0.2 M cacodylate buffer and transferred into 0.2 M sodium cacodylate with 1.0% osmium tetroxide (pH 7.2) for 2 h at room temperature and then washed 3 times with buffer. The specimens were then dehydrated in a graded series of ethyl alcohol (30%, 50%, 70%, 90%) for 15 min exchanges, and then into 100% ethanol for two fifteen min exchanges. The

specimens, in 100% ethanol, were then transferred to a Tousimis 810 critical point dryer and critically point dried with liquid CO_2. The dried tissue was mounted on specimen stubs with a silver conductive paint and sputter coated with gold-palladium with a Technics Hummer VI sputter coater and examined with a JEOL T-200 SEM. Final images were recorded with Polaroid Typd 55 P/N film.

Transmission electron microscopy (TEM)

Plaque debris obtained from each patient was dispersed in 1 ml of sodium phosphotungstate, pH 7.3. A drop of the suspension was placed onto a formvar-carbon coated 300 mesh copper grid and examined in a Siemens IA electron microscope at 80 kV. Electron micrographs were recorded on Kodak electron image plates.

Each sample examined in the TEM was evaluated for the types of spirochetes present based on the number of axial filaments originating from each end of the cell. In each case, a minimum of 20 spirochetes was counted per sample and the number of filaments and their arrangement recorded. During this analysis selected electron micrographs were taken to record the types of spirochetes found in the samples.

Preparation of Antisera

Specific immune rabbit sera is being prepared in New Zealand White rabbits for each of the spirochete strains listed in Table 29 as well as any future isolates. The protocol used for preparing antiserum for these strains consists of weekly intravenous inoculations of 2.0 ml of a 6 to 7 day old broth culture for 4 weeks. Five days after the last immunization, blood is collected by cardiac puncture on three successive days and the serum separated and the three bleedings pooled. Microscopic agglutination tests (MA) are done to determine the titer of the pooled antiserum. In the past we have obtained MA titers of 1:10,000 or greater using this immunization schedule.

Serological Relationships Among Spirochete Isolates

All of the spirochete isolates are grown in the appropriate liquid medium and the cell density adjusted to approximately 1×10^8 cells/ml. These cell suspensions are then

used to perform reciprocal adsorption microscopic agglutination studies similar to that described by Kemeny et al. (67) for Leptospira for determining the degree of antigenic cross-reactivity. We have previously published the results of similar studies with three of our isolates of T. denticola, strains 11, W and 14 (56). Thus we feel that this is the appropriate way to proceed to determine antigenic relatedness. With this approach, we will be able to determine different serotypes. The definition of a serotype will be based upon the criteria established by the World Health Organization for the classification of Leptospira, which states: "two strains are considered to belong to different serotypes if, after cross-adsorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remains in each of the two antisera in repeated tests" (68).

Sodium Dodecyl sulfate-Polyacrylamide Gel Electrophoretic Analysis of Spirochetal Antigens (SDS-PAGE)

The SDS-PAGE analysis of our isolates was performed in the following manner. The gels and solutions used were modifications of those previously described by Swindlehurst, et al. (69), and Payne (70). The spirochete isolates were grown in 50 ml broth cultures for 6-7 days, harvested by centrifugation at 10,000 x g for 20 min. The pellets suspended in 0.1 M Tris buffer, pH 6.8, to an OD_{650} of 1.0 as measured in a Spectronic 20 spectrophotometer. One hundred µl of each sample was added to a tube containing 100 µl of a solution of Tris, SDS, 2-mercaptoethanol, sucrose and Bromophenol blue to bring the sample to a final concentration of 0.0625 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% sucrose and 0.001% Bromophenol blue. The SDS-cell suspension was then heated in a boiling water bath for 2 min, cooled to room temperature and applied directly to the gel. Thirty µl of the SDS-cell suspension were added to the wells of a vertical 10 cm x 14 cm x 1.55 mm discontinuous polyacrylamide gel slab composed of a 5% acrylamide (2.5 cm x 14 cm) stacking gel (0.125 M Tris HCl pH 6.8) and a 12.5% acrylamide separating gel (0.375 M Tris HCl, pH 8.8). Using a Bethesda

Research Laboratories (BRL) vertical gel electrophoresis system (Model V161) and an A. H. Thomas electrophoresis power supply (Model 121) set at 50 mA (constant current), electrophoresis was carried out in a 0.025 M Tris-0.192 M glycerine buffer, pH 8.3, until the Bromophenol blue tracking dye reached the bottom of the gel.

The gel was removed from its glass plate sandwich and fixed in an aqueous solution containing 10% trichloroacetic acid, 5% sulfosalicylic acid (W, W, V) for 1 h. The fixative was then removed and the gel allowed to equilibrate in an aqueous solution of 25% methanol, 5% acetic acid (V, V, V) for 30 min. Following equilibration, the gel was then stained for 6 h in 0.1% Coomassie Brilliant R-250 Blue in an aqueous solution of 25% methanol and 5% acetic acid and then destained in an aqueous solution of 25% methanol, 5% acetic acid until the background was clear. Once the gels were destained, they were photographed with Polaroid 55 P/N film to record the gel patterns.

Results

Microscopic Analysis

Dark-field microscopic analysis of ANUG plaque samples revealed an abundance of rods, cocci, and spirochetes. Counts of the numbers of each morphological type in the plaque samples with the Petroff-Hausser counting chamber results in the percent composition of each type as shown in Table 27 for each of the 17 samples evaluated. Permanent video tape records of the morphological types seen under dark-field and phase contrast microscopy were also prepared using television photomicrography for selected patient samples.

The distribution of types of spirochetes bases on axial filament numbers and arrangement by negative staining TEM for selected ANUG samples is shown in Table 28. The predominate spirochetes appear to be the one with the "2-4-2" axial filament arrangement which are presumable Treponema denticola. The next most abundant spirochetes observed were equally divided among the large size spirochete of the "12-24-12", "6-12-6", and "8-16-8" class. Examples of some of these types is shown in Figures 1-3.

Table 27

Percent Morphological Composition of ANUG Plaque

Sample Number	% Rods	% Cocci	% Spirochetes	Total Organisms
1	28.60	21.31	41.80	122
2	52.90	23.22	23.87	155
3	48.64	30.63	20.72	111
4	48.14	31.85	20.00	270
5	48.75	23.75	27.50	160
6	38.91	24.63	36.45	609
7	43.94	18.94	37.12	528
8	50.34	49.65	0.00	145
9	35.48	17.35	47.17	513
10	39.55	32.88	27.61	268
11	51.38	19.89	28.73	181
12	37.19	38.11	24.69	328
13	42.86	50.00	7.14	266
14	42.80	21.02	37.31	528
15	38.80	26.86	34.33	335
16	46.39	38.14	15.46	97
17	32.01	25.30	42.68	328
Avg. %	42.75%	29.02%	29.97%	4994

Table 28

Distribution of Spirochetes in ANUG Debris

	Axial Filament Arrangement							
Patient #	1-2-1	2-4-2	3-6-3	4-8-4	6-12-6	8-16-8	12-24-12	16-32-16
1	4	8	0	1	8	6	9	0
2	2	11	0	0	3	0	6	0
3	0	0	2	3	5	1	7	5
4	0	5	0	0	1	7	11	2
5	2	2	1	2	0	9	2	0
6	2	16	0	2	2	10	4	1
7	7	16	0	3	16	0	8	0
8	10	15	2	1	12	0	10	0
9	3	13	1	3	10	19	1	0
Totals	30	84	6	15	57	52	58	8
% of Total	9.7	27.0	2.0	4.8	18.4	16.8	18.7	2.6

Scanning electron microscopic analysis was performed on freshly extracted teeth from one patient who was diagnosed as having juvenile periodontitis with super imposed ANUG. Several teeth were extracted and the adherent plaque evaluated by SEM. Figure 4 is an SEM of tooth #15 showing that the predominate flora found was that of spirochetes.

Isolation and Cultivation of Spirochetes from ANUG Lesions

Significant progress has been made in isolating and cloning new spirochete strains from ANUG lesions (Fig. 5). We have been successful in isolating 10 new strains (Table 29), 5 of which have been found to be T. denticola and the remaining need to be evaluated morphologically and serologically. These ANUG isolates are currently being compared to other strains of spirochetes which we have isolated from patients with other periodontal problems or have been acquired from other investigators. Table 30 is a list of the known isolates and their axial filament arrangment, currently available in our laboratory. To date, isolated strains of MI, MS, RM, AR and DW appear to be T. denticola. Final confirmation will come for these and the other strains when the serological analysis is completed.

Serological analysis of ANUG isolates

Antisera is currently being raised against each of the isolates found in Table 29 as well as those strains obtained from other sources (Table 30). Homologous and heterologous MA analysis as well as reciprocal MA adsorption analysis is currently being performed on each of our ANUG isolates. The serological results obtained should indicate which of the isolates are similar and how those isolates obtained from ANUG patients compare serologically to those isolates obtained from patients with other forms of periodontal diseases.

SDS-PAGE analysis of ANUG isolates

Preliminary SDS-PAGE analysis has been performed on some of the ANUG spirochetal isolates. SDS-PAGE patterns obtained for each isolate has been compared

Table 29

Oral Spirochetes Isolated from ANUG Patients

Organism	Strain Designation	Axial filament Arrangement	Source
T. denticola	AR	2-4-2	ANUG
T. deneticola	MS	2-4-2	ANUG
T. denticola	DW	2-4-2	ANUG
T. denticola	MI	2-4-2	ANUG
T. denticola	RM	2-4-2	ANUG
?	22	?	ANUG
?	23	?	ANUG
?	A10211	?	ANUG
?	A10212	?	ANUG
?	A928	?	ANUG

Table 30

Spirochete Strains Available in our Laboratory for Comparative Studies

Organism (a)	Strain Designation (b)	Axial filament arrangment	Source (c)
T. denticola	T	2-4-2	AP
T. denticola	W	2-4-2	AP
T. denticola	11	2-4-2	AP
T. denticola	JZ	2-4-2	AP
T. denticola	MS	2-4-2	ANUG
T. denticola	DW	2-4-2	ANUG
T. denticola	MI	2-4-2	ANUG
T. denticola	JP428	2-4-2	GJP
T. denticola	RM	2-4-2	ANUG
T. denticola	USA	2-4-2	AP (Rhesus monkey)
T. orale	P2	1-2-1	U. MASS
T. orale	P3	1-2-1	U. MASS
T. orale	P4	1-2-1	U. MASS
T. orale	P5	1-2-1	U. MASS
T. orale	P8	1-2-1	U. MASS
T. vincentii	N-9	5-10-5	JOHNS HOPKINS U.
T. phagedenis	Reiter	5-10-5	WVU
T. phagedenis	Kazan 5	5-10-5	WVU

a. The organisms labelled T. denticola were identified as such based on axial filament arrangement, dark-field morphology and GLC analysis of metabolic end products.

The organisms labelled T. orale were identified as such based on axial filament arrangement, dark-field morphology and nutritional requirements.

b. T. denticola strains W and 11 are now ATCC #33520 and #33521 respectfully.

c. T. denticola, USA was isolated from a Rhesus monkey with periodontal disease.

T. orale strains were obtained from Dr. E. Canale-Parola, U. Mass, who had isolated them from the oral cavity of laboratory workers. The medium used had pectin as the sole source of carbon.

T. vincentii N-9 was obtained from Dr. Paul Hardy, Johns Hopkins U.

T. phagedenis strains were obtained from Dr. Nyles Charon, West Virginia University.

with those of known spirochetal strains. Figure 6 is an example of the typical gel patterns obtained using this technique. A comparison of the major and minor proteins can be made and those organisms that are identical display identical gel patterns. Lanes 1-4 are known T. denticola isolates; lanes 5-7 are unknown ANUG isolate MS, EM and 22; lanes 8-12 represent strains of T. orale; land 13 contains T. vincentii, N-9 and lanes 14 and 15 contain two non-oral spirochetes, T. phagedenis biotype Reiter and Kazan 5 respectively. As one can see strains that are identical will display identical gel patterns i.e. lanes 1 and 2 and lanes 11 and 12 whereas strains of the same species can display significant differences in protein profiles (lanes 2 and 3). The ANUG isolates chosen for this particular experiment (lanes 5-7) have distinctly different gel patterns and therefore probably represent three different strains. Additional confirmation of this should result from the serological and morphological analysis.

LEGENDS

Figure 1. Spirochetal growth obtained following 7 days incubation on PPLO-serum medium. Note the veil of growth and the holes in the agar where samples were removed for transfer and microscopic observations.

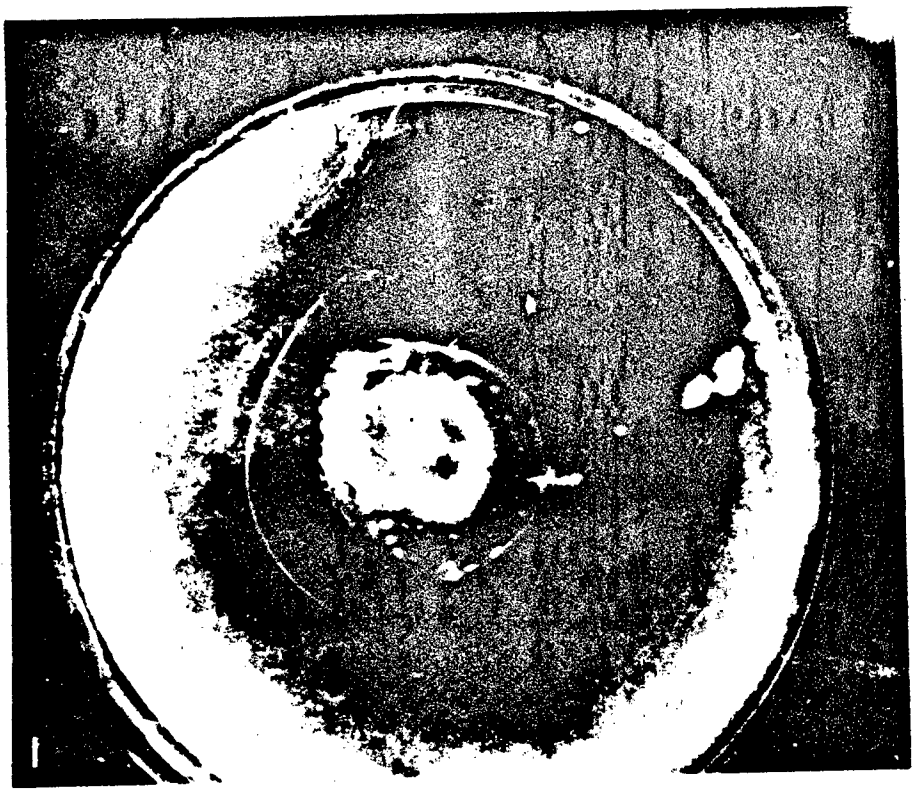
Figure 2. Electron micrograph of a negatively stained "1-2-1" oral spirochete observed in debris from an ANUG patient. X22,000

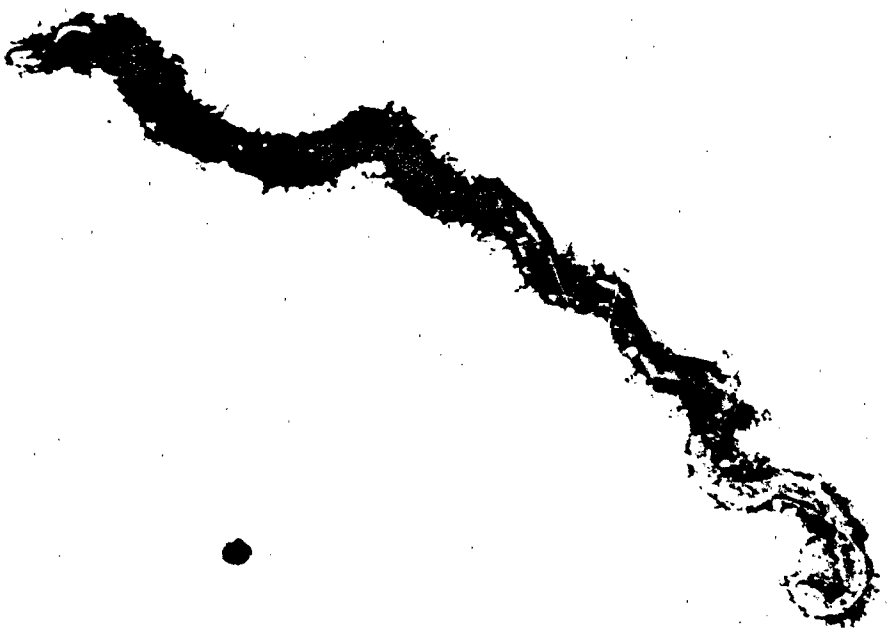
Figure 3. Electron micrograph of a negatively stained "ANUG" spirochete isolate. Note the 2-4-2 axial filament arrangements. X26,000

Figure 4. Electron micrograph of a negatively stained "8-16-8" oral spirochete observed in debris from an ANUG patient. X55,000

Figure 5. Scanning electron micrograph of the adherent plaque of an extracted tooth from a patient with ANUG. Bar marker equals 5 µm. Note the numerous spirochetes found in the specimen.

Figure 6. SDS-solubilized whole cell protein profiles of 13 oral anaerobic spirochete isolates, Treponema phagedenis biotype Reiter, and T. phagedenis biotype Kazan 5 after SDS-PAGE and staining with Coomassie Blue dye. Lanes 1-7 represent different isolates of T. denticola; lanes 8-12 represent different isolates of T. orale; lane 14 represents T. vincentii; and lanes 15 and 16 are T. phagedenis biotype Reiter and T. phagedenis biotype Kazan 5.

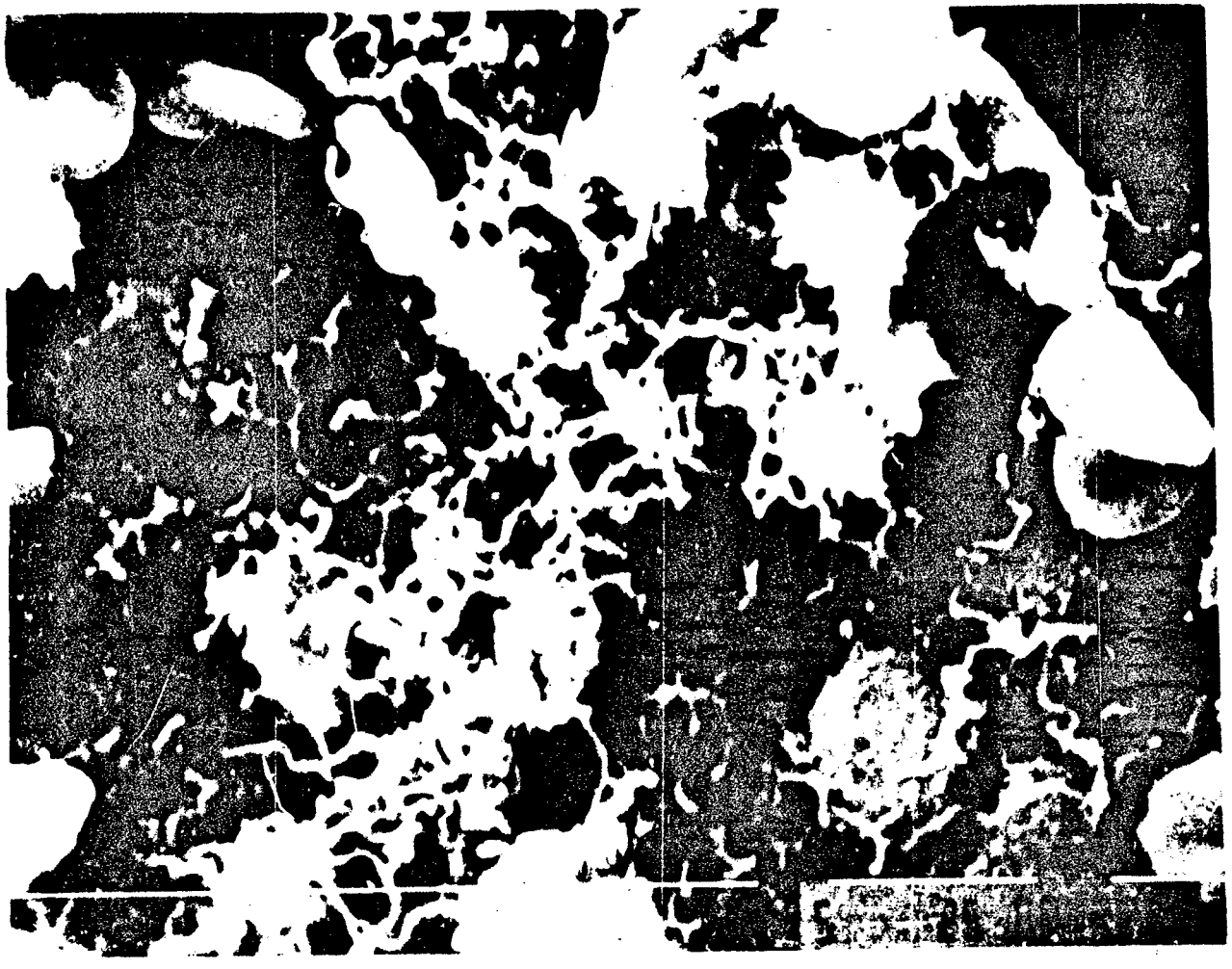


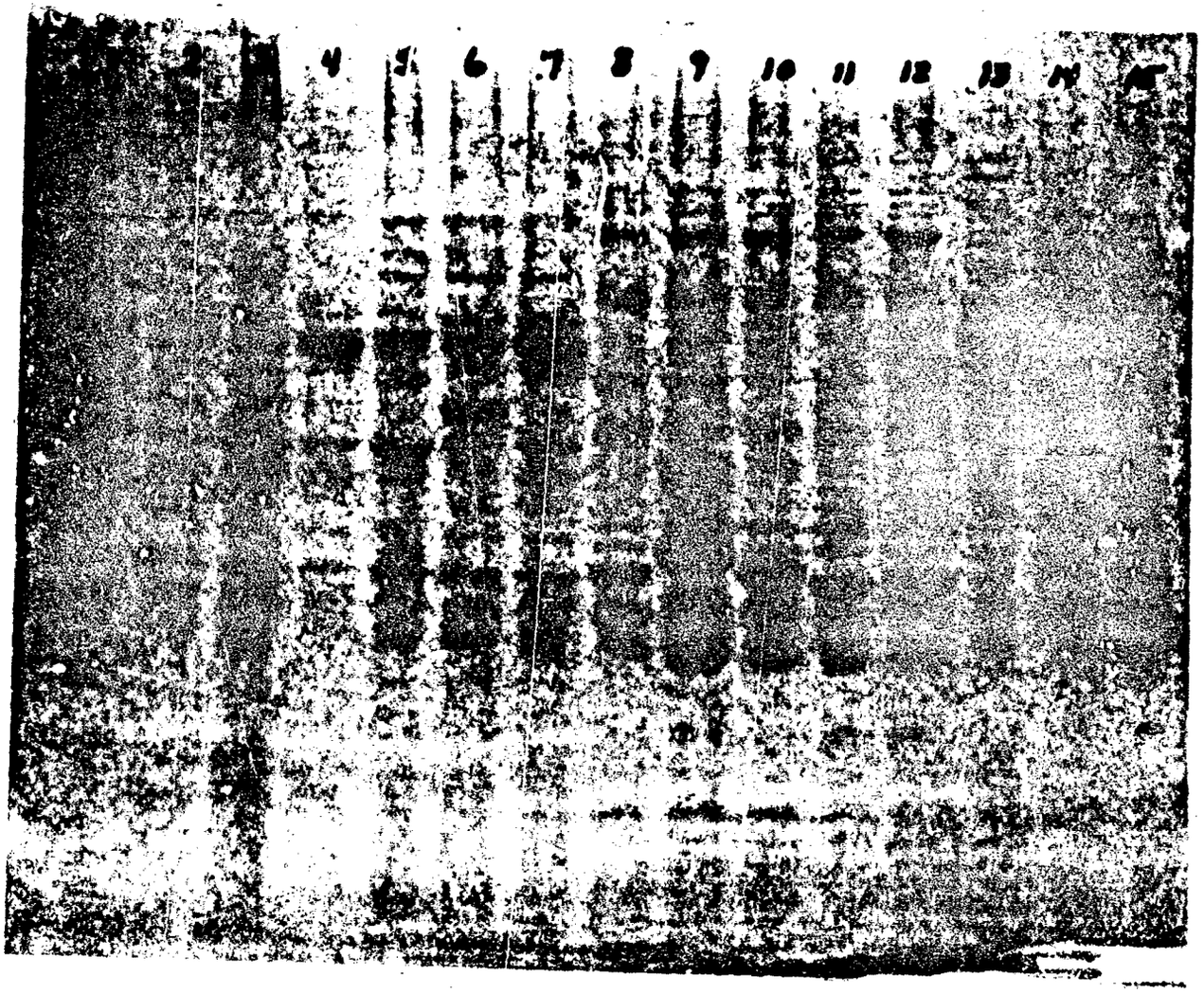


2

3

4





Interactions of Microorganisms of Acute Necrotizing Ulcerative Gingivitis and Corticosteroids

Individuals under stress and with ANUG have been shown to have higher levels than normal of corticosteroids in their serum and urine. The microorganisms which increase in number in the ANUG lesion appear to be members of the normal oral flora. It is possible that the presence of higher levels of corticosteroids in the serum may allow increased growth and or production of pathogenic factors which may participate in the formation of the ANUG lesion.

Studies were undertaken and are currently being pursued to:

1. Determine if microorganisms associated with acute necrotizing ulcerative gingivitis (ANUG) have surface receptors for human corticosteroids. The organisms to be tested are Fusobacterium nucleatum, Treponema microdentium and Bacteroides gingivalis.
2. If receptors are observed to determine if the growth of the microorganism is increased in the presence of the steroids.

MATERIALS AND METHODS

Media

The following media were used: Crystal Violet-Erythromycin (CVE) agar; MM10 agar; and Trypticase Soy Agar (TSA) with hemin and menadione (TSAHK). CVE agar (pH 7.2) contained in g/l the following: trypticase (10.0), yeast extract (5.0), NaCl (5.0), Tryptophan (0.2), agar (15.0) and crystal violet (0.005), erythromycin (0.004), and defibrinated sheep blood (50 ml) which was added after autoclaving. MM10 contained/liter: H_20 (890 ml), 37.5 ml of 0.6% K_2HPO_4, 37.5 m of a salt solution (NaCl, 1.2 g; $(NH_4)_2S0_4$, 1.2 g; KH_2PO_4, 0.6 g, Mg_2SO_4, 0.25 g and 100 ml H_20), bacto-agar (15.0 g), trypticase (2.0 g), yeast extract (0.5 g), sucrose (30.0 g), KNO_3 (0.25 g). 0.05% hemin (2.0 ml) and cysteine (0.12 g), 8% Na_2CO_3 (5 ml), DL-dithiothreitol (0.1 mg) and sheep blood (20 ml) which was added after autoclaving. TSA contained 40 g/l trypticase soy agar and for the preparation of TSAHK 10 ml of 0.5 mg/ml hemin in 0.1 N NaOH, 1 ml of 0.1% menadione in 95% ETOH and 50 ml of defibrinated sheep blood was added.

Cultures and Cultural Conditions

Strains of Bacteroides gingivalis were purchased from ATCC and VPI. They were grown in brain heart infusion (BHI) broth (BBL) supplemented with hemin (5 mg/ml) and menadione (0.2 mg/ml). The Fusobacterium nucleatum strains were grown in a modified tryptone medium. Incubation for all strains was 37° C using the BBL jar Gas-Pak system. All bacterial strains after recovery from the lyophilized state were tested for purity by subculturing on TSA (BBL) with 5% sheep blood. The colonies showing typical morphology were gram stained and the organisms observed by phase microscopy. After designated growth periods in the respective media, each bacterial culture was harvested by centrifugation at 10,000 x g for 20 min and the sedimented organisms washed x 3 in 0.01 M phosphate buffer containing 0.15 M NaCl and 0.2% sodium azide (PBS), pH 7.4. The washed sedimented organisms were resuspended in PBS to a 10% suspension and kept at 4° C until used in steroid binding assays.

Buffers

All water was deonized followed by glass distillation. All pH values were determined using a Corning No. 476050 combination electrode with Beckman No. 566002 buffer as a standard, and a Corning Model 12 pH meter. TED buffer (.01 M TRIS, .015 EDTA, 5×10^{-4} DTT), TEDG buffer (TED buffer + 10% glycerol) (v/v), and TED buffer + 30% glycerol were adjusted to pH 7.4 at 0 degrees C. The percentage of glycerol in each buffer solution was checked with a Bausch and Lomb refractometer.

Cytosol Preparation

Bacteria were washed with 10% TEDG, sonicated at maximum speed, six times for 30 sec with 10 sec cooling time in between each time. The homogenate was then centrifuged at 40 K for one hour in a Beckman T50.1 Rotor. The supernatant fraction (cytosol) was carefully poured off of the pellet. The protein content of the cytosol was determined according to the method of Lowry (14) using bovine serum albumin as a standard.

Single Saturation Dose Assay

All assays were performed in duplicate. Cytosol (0.5 ml) was incubated with a final concentration of 1×10^{-8} M labeled steroid to determine total bound steroid. Cytosol (0.5 ml) was also incubated with 1×10^{-8} M labeled steroid in the presence of 100 fold excess unlabeled steroid to determine non-specific bound steroid. Each reaction mixture was then treated with dextran-coated charcoal (DCC) to remove free steroid. Dextran-coated charcoal (0.5 ml) (5 g % Norit A, 125 g % dextran in TED buffer) was pelleted by centrifugation at 2,500 g for 20 min. The reaction mixture was pipetted on to the charcoal pellet and briefly mixed with a vortex mixer. After a 10 min incubation the mixture was again centrifuged at 2,500 g for 10 min. The supernatant fluid (0.3 ml) was layered on gradients and aliquots of 0.1 ml were removed for determination of radioactivity. Specific binding was then determined by subtracting non-specific binding

from total binding.

Scatchard Analysis

For Scatchard analysis, cytosol was incubated with steroid solutions containing radioactive steroid (for 17 -estradiol ranging from 9.6 x 10^{-11} - 2.6 x 10^{-9} M for glucocorticoids using ^{3}H-dexamethasone ranging from 3.8 x 10^{-10} - 2.7 x 10^{-9} M) for 16-24 h at 0-4 degrees C. At the end of the incubation period, the total radioactivity in the samples were measured using 10 µl samples. The free steroid was then extracted by the addition of 125 µl of a dextran-coated charcoal suspension. (1.25 g of Norit A and .625 g dextran T-40 per 100 ml TED buffer). The charcoal-cytosol mixture was incubated for 20 min at 0-4 degrees C and the charcoal was then sedimented by centrifugation at 3,000 x g for 20 min. The radioactivity in the supernatant was then be measured using 100 µl samples. The plots were corrected for nonsaturable binding.

Glycerol Density Gradient Centrifugation

Linear 10-30% glycerol gradients were prepared in 5 ml cellulose nitrate tubes using a locally constructed gradient former and a peristaltic pump (Technicon Instrument Corp., Tarrytown, New York). A portion of the cytosol (0.3 ml) was carefully layered over the gradients. The gradients were either centrifuged in a Beckman Type SW50.1 swinging bucket rotor for 16 h at 149,000 g or a Sorvall type TV865 verticle angle rotor for 2 hours at 365,000 g. Sedimentation values were determined from patterns of known standards (^{14}C BSA, ^{14}C ovalbumin, ^{14}C globulin, and catalase) run simultaneously with samples in parallel gradients. Radioactive standards were prepared by acetylation of the proteins with ^{14}C -acetic anhydride. A catalase solution was then prepared by dissolving 100 mg in 0.1 ml of TED buffer and layering 0.1 ml of the solution on the surface of the gradient. After centrifugation the absorbance of each fraction was determined at 405 nm. Fractions were collected by inserting a thin steel tube to the bottom of the gradient and removing contents by peristaltic pump. Three drop fractions (about 0.2 ml) were collected into scintillation vials using a LKB Ultrorac fraction collector (LKB, Stockholm, Sweden). Scintillation cocktail (4 ml) was added to each

tube and mixed with a vortex mixer.

RESULTS

Glycerol density gradient centrifugation profiles of cytosol obtained from Bacteroides gingivalis (CS43) were examined (Figure 7). Cytosols were incubated in 1 x 10^{-8} M (^{3}H)-dexamethasone for 22 h at 1-2° C in the presence or absence of a 100-fold excess (1 x 10^{-6} M) competing unlabelled dexamethasone or cortisol. It is evidenced by these competition studies that there exists a specific binding protein for dexamethasone in this bacteria. Concentrations of binding protein completed from the suppressible binding of (^{3}H) dexamethasone in the presence or absence of 100-fold unlabelled competition were determined (19.8 fentomoles/mg protein in 100-fold excess dexamethasone, 38.6 fentomoles/mg protein in 10-fold excess cortisol).

Similar experiments were performed with various bacteria: 10953, 10197, Fusobacterium nucleatum, XIX 9 and VI A (Table 31). No specific binding was observed in these bacteria.

DISCUSSION

Hormones are chemical signals that interact with specific target cells to promote a particular response. Steroid hormones regulate gene expression in eukaryotic cells which elects a specific response from the cell. Target cells for a particular hormone contain specialized molecules, receptors, that find the hormone and subsequently mediate its metabolic activities. A receptor has 2 roles, the first is to distinguish a particular signal from the variety of hormones and other molecules infringing on the cell, and the second is to relay this signal in such a way that the appropriate cellular response follows.

In studies with plaque flora associated with periodontal diseases, certain bacterial species are present in greater proportion than during gingival health. If periodontal disease involves increases in specific components of the indigenous population, the determination of factors which might allow or initiate this change is essential to an understanding of the etiology of the disease.

Emotional factors such as stress are associated with higher than normal levels of corticosteroids in blood serum and urine. The microorganism Bacteroides gingivalis (CS43) has been shown to possess a specific binding protein for cortisol. It is possible that the presence of higher levels of corticosteroids in the serum may allow for increased growth of these bacteria. Extensive work on the eukaryotic cell has conclusively demonstrated that specific receptors mediate metabolic processes. We have observed a high specificity glucocorticoid binding protein in these bacteria and speculate that this "receptor-like" protein is instrumental in the normal development and activity of these cells. Further studies are necessary to refine our gross characterization of this protein and qualify its functional role in the bacteria. Several variables exist in this scheme including the effect of glucocorticoid concentration on the growth of the bacteria population, rates of protein and RNA synthesis, and DNA replication. An understanding of these processes will provide an insight into the true nature of this hormone-bacteria interaction. These bacteria allow an ideal system to work with due to their high rate of turnover and general specimen availability.

Figure 7. Glycerol density gradient sedimentation patterns of Bacteroides gingivalis cytosol incubated overnight at 1-2° C with 1 x 10^{-8} (3H) dexamethasone in the presence or abscence of 100-fold excess unlabeled competing steroids. (▲) in the absence, (●) in the presence of dexamethasone, (■) in the presence of cortisol.

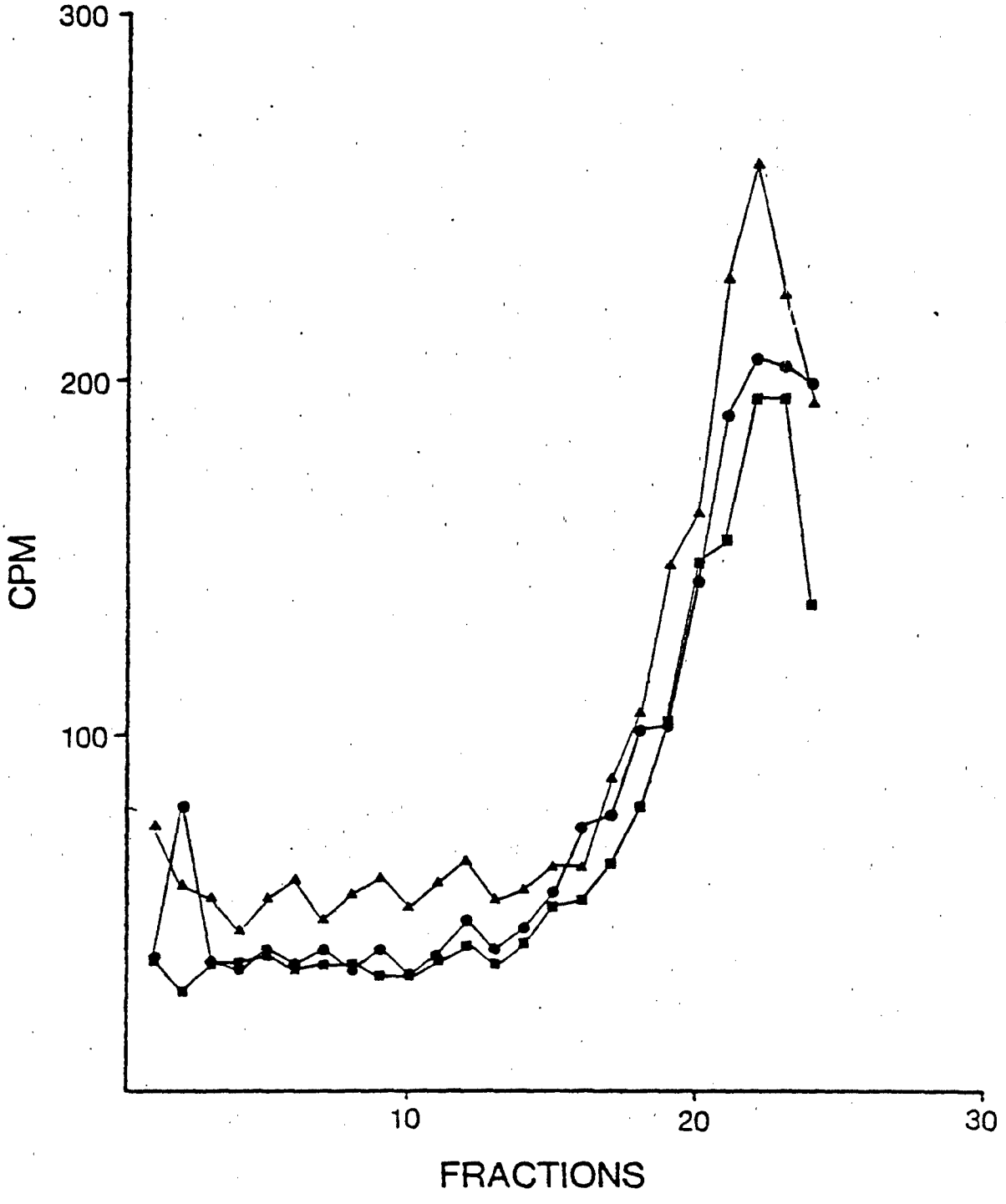

300
200
100
CPM
10
20
30
FRACTIONS

Table 31

Specifically Bound Steroid Fentomoles/Mg Protein

Bacteria	*Gluc x 10^{-6} M DEX^{-4}	Cort^{-4}	Prog^{-4}	*Prog x 10^{-6} M Prog^{-4}	R5020^{-4}	DHT^{-4}	*E_2 x 10^{-6} M DES^{-4}	P^{-4}	DHT^{-4}
CS43	++	++++	-	-	-	-	+	-	
10953	+	-	-						
10197	-	-			-		+		
CS44	-	-	-	-	+	-	-	-	
F. nuc.	-	-			-		+		
XIX-9 B. Int.	-	-			-		+		
XII-8 B.G.	-				-		-		
IV A Fuso.	-	-	-	-	+	-	+	-	+

Histopathological Studies

There is a problem obtaining biopsy tissue from ANUG patients as they will not allow removal of their gingival tissue. The biopsy specimens that we have studied (Tables 32 and 33) suggest that the type of inflammatory infiltrate is dependent upon when the biopsy was taken with respect to time after onset. As one would expect the early lesion is primarily PMNs (Table 33) and the later lesion (Table 32) shifts towards a lymphocytic infiltration. We hope to get more tissues for study and to use the tissues we have obtained for immunohistologic studies to determine the types of lymphocytes present.

Table 32

16 Y O B F

**Onset: 7 days prior

ANUG 81-1498

Overall: Predominantly lymphocytes making up cellular infiltrate with PMN's confined mostly to blood vessel (capillary) areas.

Counts: 10 random fields along ulcerated area.

	Lymph's	PMN's	Mac's/Mono	Plasma	
1	49	19	0	0	
2	39	1	2	0	
3	26	2	1	10	
4	33	9	7	1	
5	20	18	1	0	
6	9	9	0	0	
7	29	12	4	0	
8	12	35	1	0	
9	81	9	0	1	
0	94	8	1	2	(1)
Total	392	122	17	14	(545)
%	72	22	3	3	

Table 33
18 Y O B F

**Onset: 3 days prior

ANUG XVI

Overall: Predominantly PMN's making up cellular infiltrate seen primarily in ulcerated regions and overlying plaque and blood. Lymphocytes also present to less extent and interspersed amng PMN's.

Counts: 10 random fields throughout ulcerated tissue areas.

	Lymph's	PMN's	Mac's/Mono	Plasma	Mast Cell
1	11	41	0	0	0
2	8	58	1	0	1
3	1	32	0	0	0
4	7	63	0	0	0
5	29	199	1	2	0
6	11	48	0	0	0
7	10	74	2	1	0
8	0	26	0	0	0
9	9	39	1	0	0
10	14	51	2	0	0
Total: (742)	100	631	7	3	1
%	13.5	85.1	0.9	0.4	0.1

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Papers and Abstracts Resulting from this Study

PAPERS

J. W. Vincent, W. A. Falkler, Jr. and J. A. Craig. Comparison of Serologic Reactions of Typed Fusobacterium nucleatum Strains with Isolates from Humans, Canines and a Macaca mulatta Monkey. J. Clin. Micro. 1983 (in press)

W. A. Falkler, Jr., E. B. Clayman and .D F. Shaefer. Hemolysis of Human Erythrocytes by the Fusobacterium nucleatum Found in Periodontal Diseases. Archs. oral Biol, 1983 (in press)

ABSTRACTS

Serologic Studies of Fusobacterium nucleatum from Different Oral Lesions. W. A. Falkler, Jr., J. W. Vincent, R. Lai and J. B. Suzuki, 1982 IADR Meeting

Human Precipitating Antibody Reactive with Eubacterium brachy. J. W. Vincent and W. A. Falkler, Jr., 1983 AADR Meeting.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoretic Analysis of oral Spirochetes. B. D. Tall and R. K. Nauman, 1983 ASM Meeting.

Corticosteroid Receptors in Bacteroides gingivalis. W. A. Falkler, Jr., M. Salah and N. Bashirelahi. To be submitted to 1983 IADR Meeting.

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